FIOCRUZ

FUNDAÇÃO OSWALDO CRUZ

CENTRO DE PESQUISAS GONÇALO MONIZ

Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa

TESE DE DOUTORADO

O PAPEL DO ÓXIDO NÍTRICO NA PATOGÊNESE DA LEPTOSPIROSE EXPERIMENTAL EM HAMSTERS E CAMUNDONGOS

CLEITON SILVA SANTOS

Salvador – Bahia – Brasil
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CLEITON SILVA SANTOS

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Tese submetida à coordenação do Curso de Pós-Graduação em Biotecnologia em Saúde e Medicina Investigativa, Fundação Oswaldo Cruz, para a obtenção do grau de Doutor.

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" O PAPEL DE ÓXIDO NÍTRICO NA PATOGÊNESE DA LEPTOSPIROSE EXPERIMENTAL EM HAMSTERS E CAMUNDONGOS."

CLEITON SILVA SANTOS

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Dedico esta tese

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(Theodore Roosevelt).

SANTOS, Cleiton Silva. O papel do óxido nítrico na patogênese da leptospirose experimental em hamsters e camundongos. 70 f. il. Tese (Doutorado) – Fundação Oswaldo Cruz, Centro de Pesquisas Gonçalo Moniz, Salvador, 2014.

RESUMO

A patogênese da leptospirose é pouco compreendida e os estudos com enfoque na resposta inflamatória sistêmica ou local são escassos, em especial no que se refere ao papel do óxido nítrico (NO) e da enzima óxido nítrico sintase induzível (iNOS). Dados de ensaios em culturas de células renais sugerem um papel importante da liberação de mediadores inflamatórios, tais como NO, na resposta às leptospiras e consequente nefrite intersticial. A transposição destes achados para o modelo *in vivo* foi pouco explorada. Por outro lado, estudos em humanos sugerem que a liberação sistêmica de NO correlaciona-se com a gravidade da doença renal e, portanto, pode ser um potencial alvo para terapia adjuvante à antibioticoterapia. O presente trabalho estudou a correlação da iNOS com distúrbios hidroeletrolíticos na patogênese da leptospirose. No modelo experimental de hamsters, avaliamos a associação da inibição dos efeitos do NO, através da terapia combinada de antibioticoterapia padrão e azul de metileno, com distúrbios eletrolíticos, alterações histopatológicas em hamsters e sobrevida. Nenhum benefício da terapia adjuvante com azul de metileno foi demonstrado. No modelo de camundongos transgênicos, investigamos o efeito da ausência do gene da iNOS na nefrite intersticial e na quantidade de bactérias observadas nos tecidos. A ausência do gene iNOS esteve associado a menor frequência de nefrite intersticial grave. Maiores estudos são necessários para investigar a função da inibição da produção de NO na patogenia da doença e da nefrite associada a leptospirose.

Palavras-chave: Leptospirose, Modelo Animal, Azul de metileno, Óxido Nítrico Sintase

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ABSTRACT

The pathogenesis of leptospirosis is poorly understood, and there are few studies fusing on the systemic or local inflammatory responses, especially referring to the role of nitric oxide (NO) and the enzyme inducible nitric oxide synthase (iNOS). Data from *in vitro* studies suggest an important role of inflammatory mediators, such as NO, in the response to leptospires in the development of interstitial nephritis. The transposition of these findings to an *in vivo* model was little explored. However, studies in humans suggest that systemic liberation of NO is correlated with severity of renal disease and therefore can be potential target to adjuvant therapy along with antibiotic therapy. The present work evaluated the correlation of iNOS with the pathogenesis of leptospirosis. In the experimental hamster model, we evaluated the effect of inhibiting downstream effects of NO on electrolytic disorders, histopathological changes and survival. No benefit of methylene blue treatment could be observed when compared to antibiotic (ampicillin) therapy only. In the mouse transgenic model, we investigated the effect of the absence of the *Inos* gene in interstitial nephritis and in the bacterial burden in target tissues. The absence of a functional iNOS gene was associated with lower frequency of severe interstitial nephritis.

Keywords: Leptospirosis, Animal Model, Methylene blue, Nitric Oxide Synthase

LISTA DE ILUSTRAÇÕES

LISTA DE QUADROS

LISTA DE ABREVIATURAS

AMP	Ampicilina
c-NOS	Sintase do NO constitutivo
DNA	Ácido desoxirribonucleico
e-NOS	Sintase do NO endotelial
HO^-	Íon Hidróxido
IL-10	Inter leucina 10
iNOS	Sintase do NO induzível
L-NAME	NG-nitro-L-arginina-metil-éster
L-NMMA	NG-monometil-L-arginina
L-NNA	NG-nitro-L-arginina
NADPH	Nicotinamida adenina dinucleotídeo fosfato
NKCC2	Cotransportador de sódio, potássio e dois cloros
n-NOS	Sintase do NO neuronal
NO	Óxido nítrico
NONE	Nenhum tratamento
NOS	Sintase do óxido nítrico
$ONOO^-$	Peroxinitrito
Rag 1	Gene de ativação e recombinação 1
SCID	Síndrome da imunodeficiência combinada
TAL	Talidomida
TBARS	Substâncias reativas com ácido tiobarbitúrico
TNF-α	Fator α de necrose tumoral

SUMÁRIO

1. A LEPTOSPIROSE NO BRASIL E NO MUNDO

A leptospirose é uma zoonose de importância global causada por espiroquetas patogênicas do gênero *Leptospira* spp., que são transmitidas através do contato direto com a urina de animais portadores ou indiretamente pela exposição à água contaminada. A epidemiologia da doença está associada ao contato humano com roedores ou animais domésticos e selvagens (BHARTI *et al.*, 2003; MCBRIDE *et al.*, 2005). Ratos sinantrópicos são os principais reservatórios em surtos urbanos de leptospirose, que ocorrem durante estações chuvosas associadas a alagamentos em cidades com saneamento precário como em Salvador e nos demais grandes centros urbanos (KO *et al.*, 1999; RILEY *et al.*, 2007). Em outros contextos, a doença associa-se à exposição ocupacional em trabalhadores que lidam com animais de fazenda, trabalham em sistemas de esgoto ou plantadores de arroz, neste último caso sendo bem exemplificado na forma de endemias rurais no Sudeste Asiático (FAINE *et al.*, 1999). Nos países desenvolvidos, a incidência está relacionada à prática de esportes aquáticos e atividades recreacionais associadas ao contato com animais silvestre ou rios, lagos e coleções de água contaminadas. (HAAKE *et al.*, 2002; MCBRIDE *et al.*, 2005).

A infecção humana por leptospiras frequentemente tem curso assintomático e muitos pacientes apresentam quadros febris brandos e inespecíficos. A tríade clássica descrita por Weil em 1886, de icterícia, insuficiência renal e diátese hemorrágica ocorre em apenas 5-15% dos pacientes (FAINE *et al.*, 1999; BHARTI *et al.*, 2003). Nesta forma clínica, a letalidade persiste em torno de 10-15% a despeito de tratamento antimicrobiano e de suporte adequado (KO *et al.*, 1999; MCBRIDE *et al.*, 2005). A insuficiência renal aguda na leptospirose tipicamente apresenta-se de forma paradoxal não-oligúrica e hipocalêmica (SEGURO *et al.*, 1990), na qual a perda renal de sódio e potássio pode permanecer alta a despeito de grave desidratação (COVIC *et al.*, 2003). Este distúrbio pode levar à perda de volume complicada com necrose tubular aguda (COVIC *et al.*, 2003) evoluindo para o quadro de oligúria e hipercalemia, ambos marcadores bem estabelecidos de prognóstico ruim na síndrome de Weil (DUPONT, HERVE *et al.*, 1997; DAHER, ELIZABETH *et al.*, 1999; KO *et al.*, 1999;

LOPES, ANTONIO ALBERTO *et al.*, 2001; PANAPHUT *et al.*, 2002; ESEN *et al.*, 2004). A partir de 2003 a identificação da hemorragia pulmonar em pacientes com leptospirose grave despertou o interesse da comunidade científica internacional, pois, esta forma clínica passou a ser a principal causa de óbito em diversas regiões do mundo (TREVEJO *et al.*, 1998; MCBRIDE *et al.*, 2005; SPICHLER, ATHANAZIO *et al.*, 2007). Em Salvador, Bahia, um súbito aumento nos casos de hemorragia pulmonar associados à leptospirose grave observados a partir de 2003, ainda predominava como causa de óbito em 2005 (GOUVEIA *et al.*, 2008). Após o ano de 2005, a hemorragia pulmonar associada à leptospirose grave continuava a ser observada em outras partes do mundo como em Filipinas e Tailândia ((MENDOZA *et al.*, 2013; THIPMONTREE *et al.*, 2014). Segundo Mcbride e colaboradores, a hemorragia pulmonar na leptospirose grave tem desfecho letal em até 50% dos casos (MCBRIDE *et al.*, 2005).

A leptospirose assemelha-se a sepse bacteriana em alguns aspectos incluindo as manifestações toxêmicas e no padrão de resposta hemodinâmica nas formas graves (SIRIWANIJ *et al.*, 2005). No entanto, o lipopolissacarídeo da membrana externa de leptospiras tem efeito biológico 10-12 vezes menor que seu correspondente em bactérias Gram negativas (FAINE *et al.*, 1999; WERTS *et al.*, 2001). Além disso, a diátese hemorrágica que acompanha a leptospirose relaciona-se com trombocitopenia, mas não pode ser explicada por um processo de coagulação intravascular disseminada tal como na sepse (EDWARDS *et al.*, 1986; NICODEMO *et al.*, 1990; NALLY *et al.*, 2004; YANG, H. L. *et al.*, 2006). O óxido nítrico (NO) é um mediador importante da resposta vascular de pacientes com sepse (CHANDRA *et al.*, 2006), e produtos de leptospiras podem induzir células renais a expressar a enzima óxido nítrico sintase induzível (iNOS) (YANG, C.-W. *et al.*, 2002; YANG, C. W. *et al.*, 2006). Numa série de pacientes com leptospirose grave em Salvador, observamos uma associação entre os níveis séricos de NO e a gravidade da doença renal correlacionada com os níveis de creatinina séricos (MACIEL *et al.*, 2006). O papel da relação entre óxido nítrico e a patogênese da doença, portanto, precisa ser melhor estudado.

1.1 PATOLOGIA RENAL DA LEPTOSPIROSE

A patologia renal na leptospirose é tradicionalmente descrita como uma combinação entre lesão tubular aguda e nefrite intersticial (SEGURO *et al.*, 1990; FAINE *et al.*, 1999; LOMAR *et al.*, 2005; SALKADE *et al.*, 2005). No entanto, a inflamação aparentemente é um evento tardio no envolvimento renal. Numa série de 33 autópsias, alterações degenerativas tubulares foram observadas em pacientes que morreram na primeira semana de evolução; necrose tubular foi documentada nos pacientes mortos na segunda semana; e um quadro de nefrite intersticial difusa foi observado nos pacientes que morreram a partir da terceira semana de doença (AREAN, 1962). Em recente estudo experimental, observamos que, em hamsters com doença aguda letal, a nefrite só é observada em animais infectados com inóculos menores que 10^6 como 10^3 leptospiras/ml e com maior intervalo de tempo para o óbito (SPICHLER, KO *et al.*, 2007). Da mesma forma, hamsters com doença letal em experimentos de dose letal 50%, infectados com *Leptospira interrogans* sorovar Copenhageni apresentaram quadro dominante de tumefação multifocal do epitélio tubular com mínimo infiltrado inflamatório, com predomínio de macrófagos e linfócitos enquanto uma intensa nefrite intersticial foi observada apenas nos animais convalescentes necropsiados 28 dias após a infecção experimental (ATHANAZIO *et al.*, 2007).

Se a inflamação renal é, portanto, um evento tardio e secundário ao dano tóxico tubular, causada pela presença de leptospiras, esta observação limita a interpretação de um grande número de estudos recentes que focam na indução de expressão de mediadores pró-inflamatórios nas células renais *in vitro* por produtos de leptospiras (YANG *et al.*, 2000; YANG, C.-W. *et al.*, 2002; YANG, C. W. *et al.*, 2006). Por outro lado, como já foi discutido, a expressão de genes pró-inflamatórios como o da iNOS pode apresentar importância para a leptospirose, não pela associação proposta destes mediadores pró-inflamatórios com a nefrite intersticial e sim pelo efeito da alta produção do NO no transporte tubular visto que o NO é um inibidor fisiológico do cotransportador Na, K, 2Cl (NKCC2) da alça de Henle. A produção local de NO pode, portanto, explicar a perda de Na e K que é característica da leptospirose (ORTIZ e GARVIN, 2002). Um complicador adicional para interpretação destes estudos *in vitro* é que vários deles usam

células murinas e não é claro até que ponto diferentes linhagens murinas realmente desenvolvem nefrite por leptospirose *in vivo* (ATHANAZIO *et al.*, 2008). A correlação entre a expressão de iNOS no tecido renal e as alterações histopatológicas ainda não tinha sido explorada na leptospirose.

1.2 O ÓXIDO NÍTRICO COMO MENSAGEIRO SISTÊMICO E RENAL

O óxido nítrico (NO) é um radical livre, incolor, inorgânico e gasoso que possui um elétron desemparelhado, constituindo uma das menores e mais simples moléculas biologicamente sintetizadas (MORRIS e BILLIAR, 1994; BECKMAN e KOPPENOL, 1996).

A síntese do NO é catalisada pela enzima NO-sintase (NOS) e ocorre na presença de nicotinamida adenina de dinucleótido de fosfato (NADPH) e oxigênio (O_2). Nesta síntese, ocorre a oxidação de um dos nitrogênios guanidino da L-arginina transformando-a em N-hidroxi-L-arginina. Em seguida, a N-hidroxi-L-arginina é transformada em L-citrulina e NO como produto (MONCADA *et al.*, 1991; MARLETTA, 1993) como mostra a figura 1.

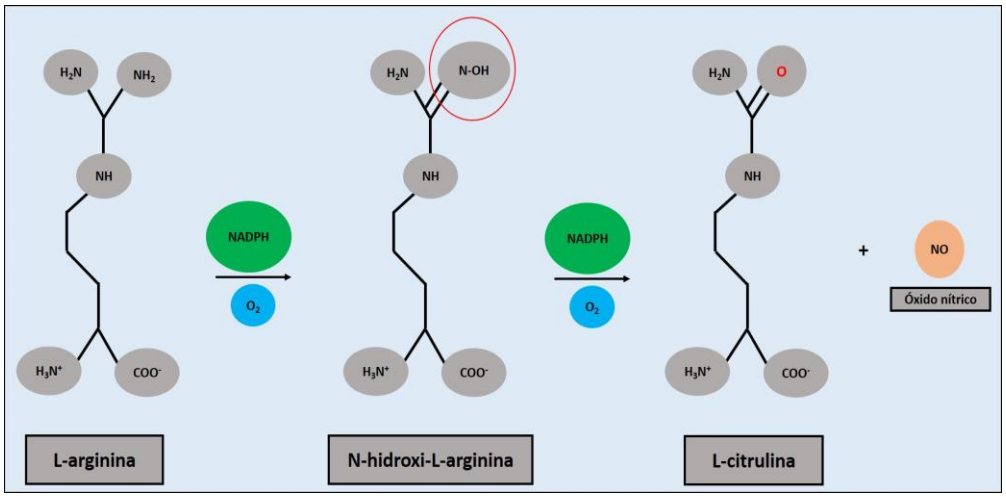

Figura 1. Reação catalisada pela NO-sintase. Adaptado de (DUSSE *et al.*, 2003).

Recentemente várias isoformas de NOS foram identificadas e agrupadas em duas categorias, a NOS constitutiva (c-NOS) que está envolvida na sinalização celular e é ativada na dependência de calmodulina e cálcio (Ca^{++}) e a NOS induzível (i-NOS) que é produzida por células do sistema imune ativadas por citocinas e por macrófagos (MONCADA *et al.*, 1991; MARLETTA, 1994).

Existem diferenças no peso molecular, na síntese de NO e na forma de ativação destas isoformas, sendo que a isoforma constitutiva compreende a NOS neuronal (n-NOS) presente nos neurônios e a NOS endotelial (e-NOS) presente nas células endoteliais. Já a forma induzível (i-NOS) que não é expressa em condições normais (BREDT e SNYDER, 1989; KNOWLES *et al.*, 1989; MONCADA *et al.*, 1991; MARLETTA, 1994). A figura 2 mostra um esquema das isoformas de NO e suas ativações.

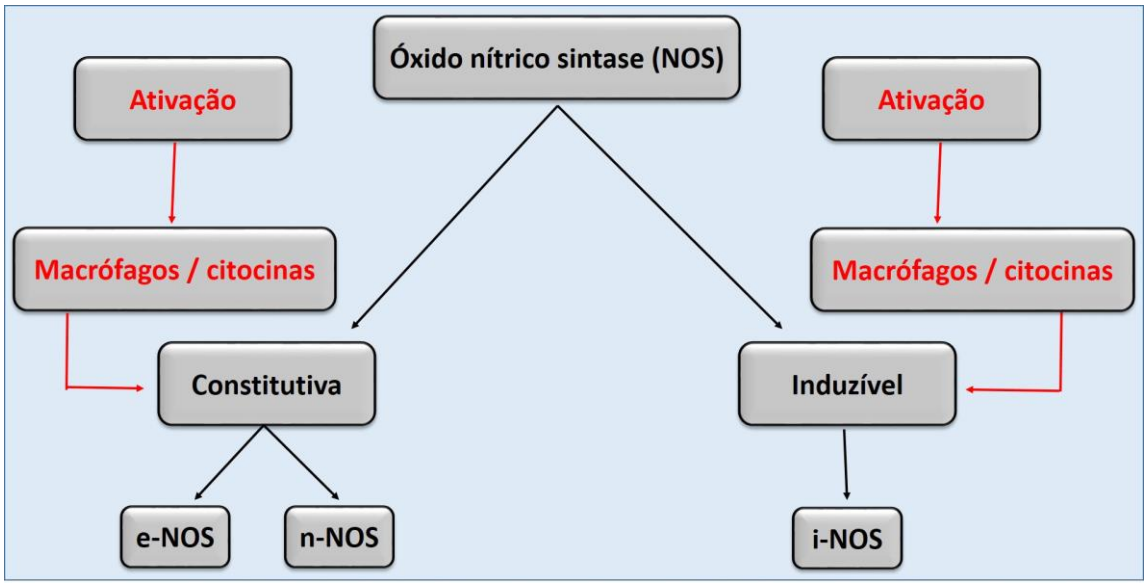

Figura 2. Esquema de isoformas de NO e suas ativações. Adaptado de (DUSSE *et al.*, 2003).

A partir da década de 80 vários estudos foram realizados com o objetivo de entender os mecanismos do NO em condições de homeostasia. Em condições normais e de homeostasia o NO produzido pela enzima e-NOS apresenta um papel importante na proteção do vaso sanguíneo como por exemplo na manutenção do tônus vascular (WENNMALM, 1994; NAVA e LUSCHER, 1995), na regulação da pressão sanguínea (NAVA e LUSCHER, 1995) e na prevenção da agregação plaquetária (VASTA *et al.*, 1995).

O NO produzido através da isoforma i-NOS exerce atividade citotóxica, que promove a destruição de micro-organismos e células tumorais (DUSSE. L. M.S., 2003). A i-NOS permanece ativada por horas após o seu estímulo devido ao seu mecanismo sinérgico da indução da produção de mais NO a partir do NO anteriormente produzido (GELLER *et al.*, 1993). E a produção descontrolada de NO pela isoforma iNOS pode levar a célula à morte (NATHAN e XIE, 1994).

Além disso, o NO reage diretamente com metais, principalmente com ferro, presente nas enzimas (MONCADA *et al.*, 1991). Em sua revisão, Dusse e colaboradores sugerem que na reação do NO com metais, incluindo o ferro, enzimas importantes para o ciclo de Krebs são desativadas, interferindo no transporte de elétrons, na síntese de DNA e na proliferação celular (DUSSE. L. M.S., 2003).

Em situações que envolvem infecção, com presença de macrófagos e neutrófilos, as células ativadas e também as células endoteliais produzem NO e intermediários reativos do oxigênio que conferem ação citotóxica. A reação entre NO e O_2 forma um poderoso oxidante de proteínas, o peroxinitrito ($ONOO^-$) que, por sua vez pode protonar com íon de hidrogênio (H^+) formando o hidroxil (HO^-). Essa reação e a formação do hidroxil aumenta ainda mais a ação tóxica do NO e O_2, podendo levar a célula produtora de NO e as células adjacentes a morte (BECKMAN e KOPPENOL, 1996).

A partir dessas evidências de que o NO pode contribuir para o agravo de diversas doenças, vários estudos têm demonstrado o efeito deletério da produção descontrolada de NO na asma (HAMID *et al.*, 1993), doenças ateroscleróticas (BUTTERY *et al.*, 1996), tuberculose (NICHOLSON *et al.*, 1996), artrite reumatoide (SAKURAI *et al.*, 1995), Alzheimer (VODOVOTZ *et al.*, 1996) e gastrite induzida por *Helicobacter pylori* (MANNICK *et al.*, 1996).

No sistema renal, o NO-mensageiro é sintetizado fisiologicamente e exerce funções importantes de homeostase, como o controle do fluxo sanguíneo e controle da excreção renal. Segundo Flora Filho e colaboradores, as três isoformas de NOS já foram identificadas nos rins (R. FLORA FILHO., 2000). A isoforma n-NOS é encontrada em todo tecido renal, estando mais concentrada na mácula densa dos rins. Já a isoforma e-NOS é mais encontrada nas arteríolas glomerulares, nos ramos aferentes e eferentes, estando mais concentrada nas arteríolas eferentes (R. FLORA FILHO., 2000).

Em sua revisão, Flora Filho e colaboradores explicam que um aspecto marcante da molécula de NO é a sua capacidade de ser benéfica ou potencialmente tóxica. Esta ação depende da sua concentração ou depuração

tecidual, estando a sua alta produção descontrolada associada com vários quadros patológicos como mostra o quadro 1 (FLORA FILHO, R.; ZILBERSTEIN, B. 2000).

Tecido	NO como mensageiro	NO como Toxina
Vasos sanguíneos	Antitrombótico, proteção à isquemia, antiaterosclerótico, inibição de proliferação do músculo liso, antiadesivo plaquetário.	Choque séptico, inflamação, síndrome de reperfusão após isquemia, extravasamento microvascular, arteriosclerose.
Coração	Perfusão coronariana, inotrópico negativo.	Choque séptico, síndrome de reperfusão após isquemia.
Pulmão	Manutenção ventilação-perfusão, motilidade bronquiolar, secreção de muco, defesa imune.	Alveolite auto imune, asma, síndrome da angustia respiratória.
Rins	Feed-back túbulo-glomerular, perfusão glomerular, secreção de renina.	Glomerulonefrite.
SNC	Memoria tardia, fluxo sanguíneo e isquemia, secreção neuroendócrina, controle visual e olfativo.	Neurotoxicidade, aumento da irritabilidade (pró-convulsivo), enxaqueca, hiperalgesia.
Pâncreas	Secreção endócrina/exócrina.	Destruição de células β.
Intestino	Fluxo sanguíneo, peristaltismo, secreção exócrina, proteção de mucosa, antimicrobiano, antiparasitário.	Dano de mucosa (hemorragia digestiva), mutagênese.
Sistema imunológico	Antimicrobiano, antiparasitário, antitumor.	Antitransplante, doença do enxerto-hospedeiro, inflamação, choque séptico, dano tissular.

Quadro 1. Efeitos do NO como mensageiro ou toxina em diversos tecidos. Adaptado de (FLORA FILHO.; ZILBERSTEIN, B. 2000).

A partir dessas evidências, diversos estudos com inibidores do NO têm sido desenvolvidos com o objetivo de amenizar os efeitos deletérios da alta produção dessa molécula nos tecidos.

A partir da identificação do NO na patogênese de algumas doenças, estudos baseados na sua inibição ou da inibição do seu precursor tem sido realizado. A L-arginina precursora da produção de NO, pode ser inibida por seus análogos como por exemplo o NG-monometil-L-arginina (L-NMMA), o NG-nitro-L-arginina (L-NNA) e o NG-nitro-L-arginina-metil-éster (L-NAME) (REES *et al.*, 1990).

Em seu estudo, Bachmann e colaboradores mostram que o bloqueio experimental da produção de NO-mensageiro promove a diminuição da irrigação renal e queda da eliminação de sódio (BACHMANN e MUNDEL, 1994).

1.3 RESPOSTA INFLAMATÓRIA SISTÊMICA EM PACIENTES COM LEPTOSPIROSE

O conceito de sepse como resposta inflamatória sistêmica na vigência de uma infecção grave vem sendo debatido recentemente, visto que nem todos os pacientes com o quadro denominado de sepse apresentam resposta imune exacerbada com liberação de citocinas pró-inflamatórias (HOTCHKISS e KARL, 2003). No entanto, pelo menos um subgrupo de quadros sépticos, notadamente a meningococcemia, de evolução tipicamente fulminante, pode ser caracterizada de modo convincente como uma “tempestade de citocinas”. Neste grupo particular de infecções, a dosagem sérica de fator de necrose tumoral alfa (TNF-α) serve como um preditor de evolução fatal (HOTCHKISS e KARL, 2003).

Os dados sobre resposta inflamatória sistêmica na leptospirose são limitados. Apesar da leptospirose ser frequentemente tratada como uma vasculite sistêmica, a inflamação na parede de vasos não é um achado comum e a tentativa experimental de reproduzir lesões vasculares demonstrou alterações endoteliais precoces (DE BRITO *et al.*, 1979). Este quadro explicaria um distúrbio de permeabilidade e aproximaria a leptospirose do que é observado na sepse. A avaliação da relação entre liberação sistêmica de citocinas e doença é, entretanto, restrita. Em estudo realizado com 18 pacientes em São Paulo, foi sugerida uma associação entre detecção sérica de TNF-α com gravidade de doença. Neste relato, apenas quatro pacientes apresentaram níveis detectáveis da citocina no soro por ELISA e três deles evoluíram para o óbito. Este estudo teve como limitação a pequena amostra e o fato de dois casos fatais terem o diagnóstico de leptospirose apenas com base em critérios clínicos e epidemiológicos, sem confirmação laboratorial (TAJIKI e SALOMAO, 1996). Numa avaliação subsequente de 12 pacientes, foi sugerida uma associação entre a maior razão de IL-10 / TNF-α e melhor prognóstico. Novamente, como limitação, este estudo teve apenas amostra de 12 pacientes e o critério laboratorial do título de microaglutinação foi de 1:100, não sendo específico para um grande centro urbano brasileiro onde a doença é provavelmente endêmica (TAJIKI *et al.*, 1997)**.** Recentemente, nosso grupo observou a elevação de outros marcadores de resposta inflamatória sistêmica como o óxido nítrico, avaliado e uma série de 35

pacientes com leptospirose, (MACIEL *et al.*, 2006) e a presença do marcador de estresse oxidativo TBARS (substâncias reativas com ácido tiobarbitúrico – marcadores de peroxidação lipídica) elevado nas formas graves de leptospirose experimental (SPICHLER, KO *et al.*, 2007).

1.4 ÓXIDO NÍTRICO E DISTÚRBIOS HIDROELETROLÍTICOS

A capacidade de produtos derivados de leptospiras estimularem a expressão de iNOS em células renais *in vitro* é bem demonstrada (YANG, C. W. *et al.*, 2002; YANG, C. W. *et al.*, 2006). Mais recentemente, foi demonstrado que a expressão de iNOS no tecido renal e as concentrações de óxido nítrico no soro de hamsters e camundongos C3H/HeJ estavam aumentadas após a infecção por leptospiras patogênicas (PRETRE et al. 2011). A alta produção de NO no tecido renal é uma hipótese possível para explicar a disfunção renal observada em pacientes com leptospirose.

O óxido nítrico é um inibidor fisiológico do co-transportador Na^+, K^+,$2Cl^-$ (NKCC2) do ramo espesso da alça de Henle (BELTOWSKI *et al.*, 2003), que tem papel fundamental na reabsorção do sódio e potássio no néfron. A inibição do NKCC2 é um mecanismo postulado que explicaria o quadro de perda urinária excessiva de sódio e potássio na leptospirose. Estudos avaliando a função urinária em pacientes com leptospirose em Taiwan, em especial nos testes de *clearance* sem resposta à furosemida (LIN *et al.*, 1999; WU *et al.*, 2004) e de experimentos *in vitro* que demonstram a exposição à membrana externa de *Leptospira* resulta em menor expressão do gene *NKCC2* (WU *et al.*, 2004) corroboraram com o mecanismo postulado. Recentemente, observamos a redução de expressão do trocador Na^+-H^+ do túbulo proximal e do NKCC2 da alça de Henle durante a leptospirose experimental grave no modelo de hamster. Estas alterações foram revertidas com uso de terapia antimicrobiana com ampicilina (SPICHLER, KO *et al.*, 2007). O mecanismo postulado de perda urinária de sódio, potássio e magnésio pela inibição do NKCC2 está esquematizado na figura 3.

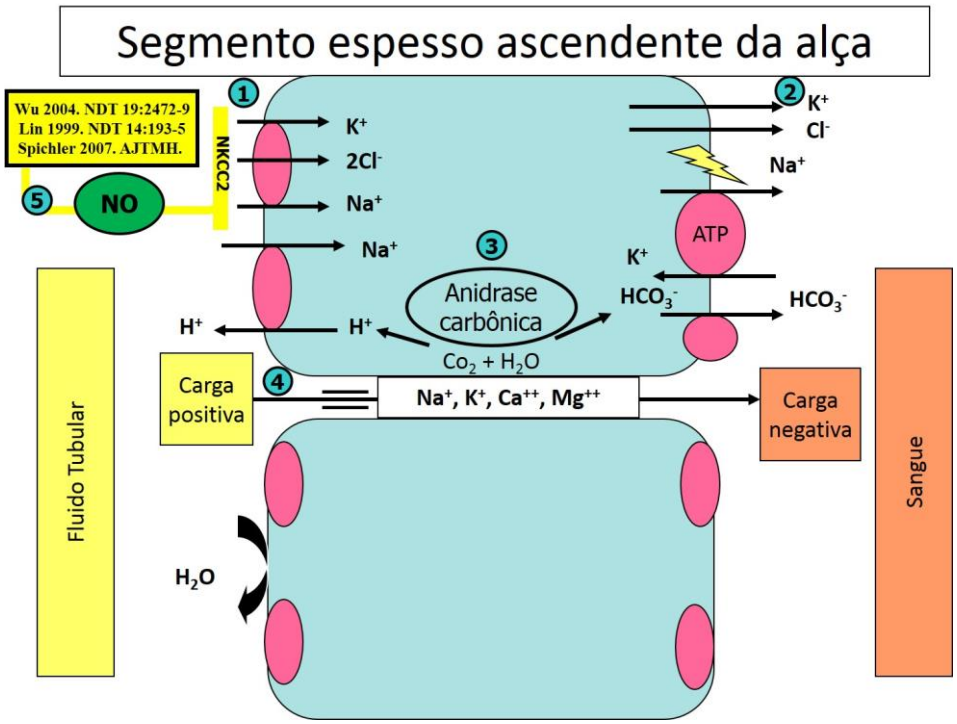

Figura 3. Esquema de reabsorção de íons, inibição do NKCC2 e a consequente perda renal de sódio, potássio e magnésio no segmento espesso ascendente da alça de henle. 1. O cotrasportador de Na+/K+/2Cl- (NKCC2) atua transportando esses íons da luz dos túbulos renais para dentro da célula. 2. Esses íons são transportados de dentro da célula para o interstício através da bomba de Na+/K+ com gasto de energia. 3. A cadeia da anidrase carbônica contribui doando H+ e HCO3- para o ambiente com fluido tubular e para o interstício respectivamente. 4. A diferença de cargas entre os ambientes com fluido tubular e interstício possibilita a reabsorção dos demais íons como Na+/K+/Ca++ e principalmente o Mg++ de forma paracelular (entre as células). 5. A possível inibição do NKCC2 pelo óxido nítrico (NO) e, ou, por produtos de leptospiras pode fazer com que esses íons não sejam transportados para dentro da célula, e possivelmente comprometendo o transporte ativo da bomba de bomba de Na+/K+ e da reabsorção de Mg++ de forma paracelular, causando depleção desses íons.

A alça de Henle é o principal segmento do néfron para reabsorção de magnésio (65% deste íon é reabsorvido no ramo espesso) usando o transporte passivo paracelular que depende de um gradiente elétrico gerado pela ação do NKCC2. A mesma nefropatia perdedora de sódio e potássio, portanto, pode resultar em excreção aumentada de magnésio. Recentemente, esta possibilidade foi demonstrada numa série de 20 pacientes tailandeses, na qual 15 apresentaram excreção urinária aumentada de magnésio e 9 pacientes, todos com insuficiência renal, apresentaram hipomagnesemia (KHOSITSETH *et al.*, 2007). A hipomagnesemia é um fator de mau prognóstico em pacientes com quadro grave em outras doenças (BERKELHAMMER e BEAR, 1985). Entretanto, ainda não foi avaliado o prognóstico de pacientes com hipomagnesemia na leptospirose.

A identificação do óxido nítrico como mediador de disfunção renal oferece racional para estudo de sua inibição como terapia adjuvante na leptospirose.

1.5 USO DO AZUL DE METILENO

O azul de metileno é um composto de fenotiazida que possui propriedades biológicas. Estas propriedades foram pesquisadas por mais de 120 anos perdurando até os dias de hoje (BRUCHEY e GONZALEZ-LIMA, 2008). O azul de metileno foi sintetizado pela primeira vez em 1876 e é tradicionalmente usado como corante, no entanto, já foi usado como opção terapêutica em diversas doenças (WAINWRIGHT e CROSSLEY, 2002); (NAYLOR *et al.*, 1986; DEUTSCH *et al.*, 1997; PELGRIMS *et al.*, 2000; WAINWRIGHT e CROSSLEY, 2002). O azul de metileno passou a ser mais conhecido a partir do seu uso em doenças como metahemoglobinemia (NEE e FITZGERALD, ; GOULON *et al.*, 1966), encefalopatia (PELGRIMS et al., 2000) e choque séptico (WEINGARTNER et al., 1999). O seu mecanismo de ação ocorre através de ligação com a enzima guanilato ciclase solúvel com consequente inibição dos efeitos biológicos da produção de NO. Além disso, a inibição do óxido nítrico contribui para a redução dos riscos de hipotensão e choque (SALARIS et al., 1991; KEANEY et al., 1994; WEINGARTNER et al., 1999). A figura 4 mostra esquema da via de produção do NO na célula endotelial e a consequente vasodilatação causada pela ação da guanosina monofosfato.

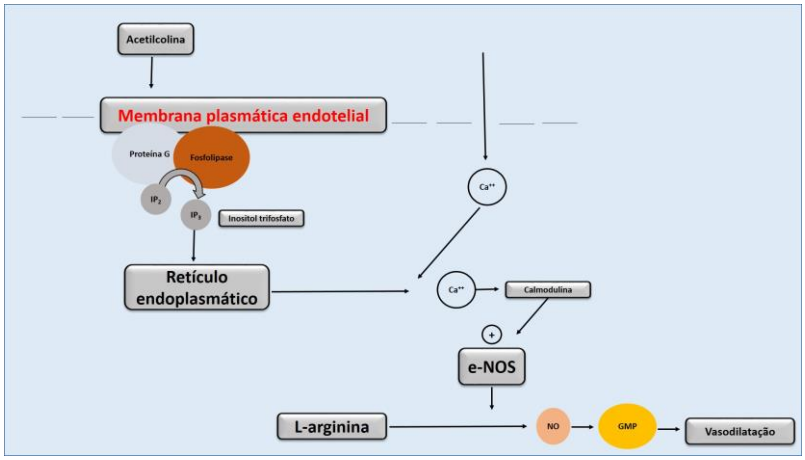

Figura 4. Esquema da produção do óxido nítrico na célula endotelial e a consequente vasodilatação através do aumento da produção de guanosina monofosfato. Adaptado de (DOS SANTOS *et al*., 2010).

O azul de metileno possui efeitos hemodinâmicos benéficos na sepse (KIROV et al., 2001; HEEMSKERK et al., 2007) tornando-o um candidato a tratamento adjuvante à antibioticoterapia na leptospirose. Esta é uma questão importante tendo em vista que a doença permanece com alta letalidade mesmo com uso de antibióticos. Esta falha terapêutica foi relacionada com o impacto reduzido dos antimicrobianos sobre o curso da doença quando iniciado o tratamento após quatro dias do início dos sintomas (W.H.O., 2003).

Recentemente, avaliamos uma terapia antioxidante utilizando N-acetil-cisteína na leptospirose experimental em hamsters, porém este protocolo de tratamento não mostrou nenhum efeito adicional em relação ao tratamento com ampicilina (SPICHLER, KO *et al.*, 2007). Apesar do resultado ruim, esta avaliação foi útil para padronizar o modelo de teste de terapia adjuvante à antibioticoterapia padrão (ampicilina) em hamsters. Neste modelo testamos terapias adjuvantes tendo como alvos moleculares o NO (inibição com azul de metileno) e o TNF-α (inibição com talidomida).

2 JUSTIFICATIVA

Na prática clínica, a leptospirose é suspeitada ou diagnosticada tardiamente, pois é frequentemente confundida com outras doenças infecciosas agudas e faltam testes rápidos para sua confirmação laboratorial. Neste contexto, o efeito de antibióticos é limitado quando o tratamento é iniciado após quatro dias de sintomas. Por este motivo, a letalidade das formas graves permanece elevada, sendo necessário a busca urgente de terapias adjuvantes aos antibióticos. Além disso, estudos já mostraram correlação entre altos níveis de NO séricos e a gravidade da doença renal em pacientes com leptospirose grave, que o NO é inibidor fisiológico da NKCC2, que o uso de azul de metileno tem efeitos benéficos na sepse experimental e em ensaios clínicos, pois, a leptospirose assemelha-se a sepse bacteriana e alguns aspectos. A disfunção do NKCC2 resulta em distúrbios iônicos incluindo a depleção de potássio, sódio e magnésio. A hipomagnesemia ainda não foi estudada em modelo experimental. O modelo de tratamento tardio tem o objetivo de simular a situação de pacientes que chegam em fase avançada nos hospitais, ou de diagnóstico tardio, para quem o bloqueio dos efeitos do NO potencialmente pode prevenir distúrbios iônicos e prolongar sua sobrevida.

3 HIPÓTESE

O tratamento adjuvante com azul de metileno, inibidor de efeitos a jusante (*downstream*) de óxido nítrico, prolonga a sobrevida e previne distúrbios iônicos na leptospirose experimental.

4 OBJETIVOS

4.1 OBJETIVO GERAL

Avaliar o papel do óxido nítrico na patogênese do envolvimento renal e pulmonar da leptospirose experimental em hamsters e camundongos.

4.2 OBJETIVOS ESPECÍFICOS

- Estudar o efeito de um inibidor da guanilato ciclase (*downstream* à iNOS), o azul de metileno, no desfecho da sobrevida, lesões renais e distúrbios hidroeletrolíticos em hamsters infectados e tratados com dose padrão de ampicilina.
- Avaliar se a ausência do gene da iNOS está relacionada com o menor grau de nefrite intersticial e a quantidade de leptospiras nos tecidos de camundongos C57/BL6.

5 RESULTADOS

Os resultados desta tese encontram-se apresentados no capítulo 1 na forma de artigo publicado. Os artigos em anexos representam trabalhos em colaboração produzidos no período do curso de pós-graduação que estão ligados indiretamente a esta tese. O anexo 1 apresenta artigo publicado que aborda a ausência do gene da *Inos* e maior frequência de nefrite intersticial em camundongos e responde ao segundo objetivo específico desta tese. O anexo 2 trata-se de um trabalho que segue a mesma lógica do trabalho apresentado no capítulo 1. Neste estudo, testamos outro tratamento adjuvante (talidomida) associado a antibioticoterapia padrão utilizando ampicilina na leptospirose experimental em hamsters. O anexo 3 valida técnicas de quantificação de *Leptospira in situ* usadas pelo nosso grupo.

Os trabalhos publicados tem os seguintes títulos: (capítulo 1) Ionic imbalance and lack of effect of adjuvant treatment with methylene blue in the hamster model of leptospirosis; (Anexo 1) Attenuated nephritis in inducible nitric oxide synthase-knockout C57BL/6 mice and pulmonary haemorrhage in CB17 SCID and recombination activating gene 1-knockout C57BL/6 mice infected with *Leptospira interrogans*; (Anexo 2) Immunomodulatory treatment with thalidomide in experimental leptospirosis in *Golden Syrian* hamsters (*Mesocricetus auratus*); (Anexo 3) Detection and Quantification of *Leptospira interrogans* in Hamster and Rat Kidney Samples: Immunofluorescent Imprints versus Real-time PCR.

5.1 CAPÍTULO 1 – ARTIGO PUBLICADO

Artigo publicado na revista Memórias do Instituto Oswaldo Cruz

Ionic imbalance and lack of effect of adjuvant treatment with methylene blue in the hamster model of leptospirosis

Cleiton Silva Santos, Everton Cruz de Azevedo, Luciane Marieta Soares, Magda Oliveira Seixas Carvalho, Andréia Carvalho dos Santos, Adenizar Delgado das Chagas Jr, Caroline Luane Ribeiro da Silva, Ursula Maira Russo Chagas, Mitermayer Galvão dos Reis, Daniel Abensur Athanazio. **Mem Inst Oswaldo Cruz**. Jun 2013; 108(4): 438–445.

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A patogênese da leptospirose e os estudos com foco na resposta inflamatória sistêmica ou local ainda são escassos. Neste trabalho avaliamos o desequilíbrio iônico e o efeito adjuvante do azul de metileno no modelo hamster de leptospirose experimental. Inicialmente foi estabelecido o modelo de tratamento tardio com início no décimo dia após a infecção. Para avaliação do desequilíbrio iônico na leptospirose foram infectados 80 hamsters, posteriormente separados em 4 grupos de 20 animais de acordo com o tratamento: Ampicilina, azul de metileno, ampicilina + azul de metileno e controles não tratados. No décimo dia após a infecção os grupos de animais infectados foram tratados por 5 dias com os protocolos pré estabelecidos para cada grupo, com exceção do grupo de animais controles não tratados. Foram realizadas necropsias nos dias 4, 8, 16 e 28 após a infecção com coleta de soro para dosagem de íons, creatinina e amostra renal para histopatologia. A análise dos resultados mostrou que a hipocalemia observada em pacientes com leptospirose não foi reproduzida no modelo hamster, pois, neste modelo todos os grupos de animais infectados apresentaram níveis séricos significativamente altos quando comparados com os animais não infectados. A hipomagnesemia não foi observada neste modelo. O tratamento adjuvante com azul de metileno não mostrou efeito sobre o potássio e o magnésio séricos na fase aguda da doença. A elevação acelerada da creatinina sérica foi prevenida com tratamento antimicrobiano no modelo hamster. O tratamento adjuvante com um inibidor da guanilato ciclase (com ação a jusante de todas Óxido Nítrico Sintases) não interferiu nos distúrbios iônicos observados e não prolongou a sobrevida dos animais no modelo de início tardio de antibioticoterapia.

Ionic imbalance and lack of effect of adjuvant treatment with methylene blue in the hamster model of leptospirosis

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Leptospirosis in humans usually involves hypokalaemia and hypomagnesaemia and the putative mechanism underlying such ionic imbalances may be related to nitric oxide (NO) production. We previously demonstrated the correlation between serum levels of NO and the severity of renal disease in patients with severe leptospirosis. Methylene blue inhibits soluble guanylyl cyclase (downstream of the action of any NO synthase isoforms) and was recently reported to have beneficial effects on clinical and experimental sepsis. We investigated the occurrence of serum ionic changes in experimental leptospirosis at various time points (4, 8, 16 and 28 days) in a hamster model. We also determined the effect of methylene blue treatment when administered as an adjuvant therapy, combined with late initiation of standard antibiotic (ampicillin) treatment. Hypokalaemia was not reproduced in this model: all of the groups developed increased levels of serum potassium (K). Furthermore, hypermagnesaemia, rather than magnesium (Mg) depletion, was observed in this hamster model of acute infection. These findings may be associated with an accelerated progression to acute renal failure. Adjuvant treatment with methylene blue had no effect on survival or serum Mg and K levels during acute-phase leptospirosis in hamsters.

Key words: leptospirosis - methylene blue - models - animal

Leptospirosis is a widespread zoonosis in which the most important and life-threatening complications are acute renal failure and pulmonary haemorrhage (Cerqueira et al. 2008, Medeiros et al. 2010). The beneficial effect of antibiotics against severe leptospirosis when treatment is initiated within four days after its clinical onset is undisputed. Although antimicrobial treatment is recommended even if it is delayed, the clinical benefits in this case are controversial (WHO 2003). When severe manifestations develop, the most important issue in clinical management is supportive therapy, including dialysis and mechanical ventilation (McBride et al. 2005). In clinical practice, leptospirosis is usually not initially suspected or is diagnosed late because its features overlap with other diseases (e.g., hepatitis and dengue) and because of the limited performance of confirmatory serological tests. Thus, despite aggressive supportive treatment, fatalities from severe forms of leptospirosis remain high and adjuvant therapies that, in association with antibiotics, could benefit patient outcomes are urgently needed. We previously developed a model of antibiotic therapy initiated late after the infection of hamsters. In the first evaluated adjuvant therapy, the antioxidant effects of N-acetylcysteine did not yield any additional benefit compared with ampicillin treatment alone (Spichler et al. 2007).

Leptospirosis causes a peculiar form of non-oliguric renal failure [characterised by potassium (K) depletion] that rapidly evolves to an oliguric hyperkalaemic form, indicative of a poor outcome (Cerqueira et al. 2008). Several reports have described patients with severe leptospirosis who developed hypomagnesaemia during the acute phase of the disease (Khositseth et al. 2008, Spichler et al. 2008, Craig et al. 2009). Within the kidneys, the major site of magnesium (Mg) transport is the thick ascending limb (TAL), where 65% of filtered Mg is reabsorbed, while 20-25% returns to the blood through the proximal tubule and 5-10% returns to the distal tubule (Berkelhammer & Bear 1985). Regardless of which major molecular targets of leptospirosis lead to tubular dysfunction, impaired ion transport results in sodium (Na) and K wasting (Covic et al. 2003, Cerqueira et al. 2008). The gradient of K and Na is the major driving force for the paracellular reabsorption of Mg in the TAL. Thus, some degree of Mg loss may be expected in K/Na-wasting states. Because hypomagnesaemia is a common feature of critically ill patients, is correlated with a poor prognosis and has been increasingly recognised in association with severe leptospirosis, we inferred that this ionic imbalance might represent a promising target for adjuvant therapy to treat leptospirosis. Therefore, we were interested in determining whether our hamster model of leptospirosis reproduces ionic changes, such as hypomagnesaemia or hypokalaemia and how these

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changes correlate with disease outcomes. Studies on experimental leptospirosis focusing on ionic changes are scarce and have been restricted to in vitro models (Wu et al. 2004), microperfusion analyses (Magaldi et al. 1992) and evaluation of tubular transporter expression via immunohistochemistry (Spichler et al. 2007). To the best of our knowledge, no study has previously evaluated the possible reproduction of serum Mg changes in experimental leptospirosis.

Clinical studies based on clearance tests suggest that the main tubular defect involved in leptospirosis is impaired function of the Na,2Cl,K cotransporter (NKCC2) in the TAL (Lin et al. 1999, Wu et al. 2004). In vitro, the NKCC2 of murine TAL cells can be inhibited using leptospiral outer membrane extracts (Wu et al. 2004). In our hamster model, we demonstrated that NKCC2 expression is reduced in TAL cells during acute infection and the downregulation of NKCC2 can be reversed by antimicrobial therapy (Spichler et al. 2007). Taken together, these data suggest a potential direct toxic effect of leptospires on tubular transporters. Furthermore, the renal loss of Mg, Na and K may be related to the production of nitric oxide (NO), which is a known inhibitor of NKCC2 (Ortiz & Garvin 2002, Beltowski et al. 2003). Inducible NO synthase (iNOS) is stimulated in vitro when tubular cells are exposed to leptospirally derived products (Yang et al. 2000, 2002, 2006) and we demonstrated that serum levels of NO correlate with a laboratory marker of renal dysfunction (serum creatinine) in patients (Maciel et al. 2006). Recently, Prêtre et al. (2011) reported increased expression of iNOS in vivo in the kidneys of hamsters and C3H/HeJ mice during acute infection (as determined via immunoblot and immunohistochemistry analyses) as well as elevated nitrite/nitrate concentrations in serum samples from these animals. Thus, inhibition of NO production represents a potential therapeutic target for adjuvant therapy in severe leptospirosis. Methylene blue is a known inhibitor of soluble guanylyl cyclase (which is downstream of the action of any NOS isoform) that shows encouraging results in patients with sepsis (Kirov et al. 2001, Kwok & Howes 2006, Heemskerk et al. 2008, Paciullo et al. 2010).

The aims of this study were to test the following parameters in our hamster model of leptospirosis (i) whether the acute form of the disease is associated with hypokalaemia and hypomagnesaemia and (ii) whether adjuvant therapy using methylene blue has beneficial effects on survival or ionic imbalance during acute experimental leptospirosis in hamsters.

MATERIALS AND METHODS

Bacteria - Leptospires were cultured in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco Laboratories, Detroit, MI) at 29ºC and were counted in a Petroff-Hausser counting chamber (Fisher Scientific, Pittsburgh, PA). An isolate from Brazil, *Leptospira interrogans* serovar Copenhageni strain L1-130, was used in all of the assays (Nascimento et al. 2004). This strain was passaged and re-isolated four times from the hamsters and was stored at -70ºC. Frozen aliquots were thawed and passaged in liquid medium eight times prior to use as a low-passage-number isolate in the infection experiments.

Study design - Nine-week-old female Golden Syrian hamsters [Oswaldo Cruz Foundation (Fiocruz), state of Bahia] were used in all of the experiments. The Ethical Committee of the Fiocruz approved the animal protocols employed in this study. Based on previous experiments in which an inoculum of 10^3 leptospires was found to cause 100% lethality, the interval between infection and death was estimated to be 10-14 days. In three preliminary experiments, late ampicillin treatment (100 mg/kg/bid) was tested to determine the day on which the initiation of antimicrobial therapy would yield an approximately 50% survival rate (Table I) and that day was chosen to test the adjuvant methylene blue therapy.

TABLE I

Effect of the interval between infection (*Leptostpira interrogans* strain L1130) and the initiation of ampicillin (100 mg/Kg/bid) treatment on the survival of nine-week-old hamsters

Experiment	1[a]		2[b]		3[b]	
Starting day	Deaths n/N (%)	Days to death	Deaths n/N (%)	Days to death	Deaths n/N (%)	Days to death
Untreated	ND	-	7/11 (64)	10, 10, 10, 10, 11, 11, 13	7/7 (100)	9, 9, 10, 13, 13, 13, 13
6	0/6	-	ND	-	0/7	-
7	0/6	-	ND	-	ND	-
8	0/6	-	0/7	-	0/7	-
9	2/6 (33)	10, 10	ND	-	ND	-
10	ND	-	2/6 (33)	12, 13	2/5 (40)	10, 15
11	ND	-	ND	-	ND	-
12	ND	-	3/4 (75)	12, 12, 12	ND	-

a: inoculum size, 500 leptospires; *b*: inoculum size, 1,000 leptospires; ND: not done; n/N: number of deaths/total number of evaluated hamsters.

The hamsters were inoculated intraperitoneally with 10^3 bacteria from virulent strain L1130. The experiment began with 80 infected animals that were observed and euthanised in groups of 20 on days 4, 8, 16 and 28. After treatment was initiated, the 20 animals from each time point were further divided into four groups according to the type of treatment initiated on the 10th day, which was ampicillin alone, methylene blue alone, ampicillin and methylene blue together or no treatment.

In a second experiment, hamsters were infected with a high inoculum dose of 10^6 leptospires and assigned to groups (of 9-11 animals) that were treated with ampicillin alone, methylene blue alone, ampicillin and methylene blue together or received no treatment. The treatments were planned to begin when the first death was observed. This study design was used as an alternative strategy to reproduce the late initiation of therapy during acute lethal leptospirosis.

Blood tests - Serum Na, K and Mg levels were measured using a Labmax 240 device (Labtest Diagnostica SA, Minas Gerais, Brazil). Na and K were quantified using ion-selective electrodes, while Mg was measured using a colorimetric method. Creatinine analyses were performed in serum with an immunochemistry assay (A25 system, Biosystems SA, Barcelona, Spain). The reference values presented in Figs 1-4 were obtained from previous clinical chemistry reports on laboratory hamsters (Tomson & Wardrop 1987). Four or five uninfected animals were also tested to serve as controls.

Histopathological analysis - Necropsies were performed immediately after euthanasia. Kidneys were fixed in 4% formalin and embedded in paraffin and 4-5 µm-thick sections were subjected to conventional histological analyses. Semi-quantitative estimation of interstitial nephritis was performed as previously described (Bandeira et al. 2011). Briefly, grade + nephritis was defined as an infiltrate that was rich in macrophages and lymphocytes restricted to periarterial areas, grade ++

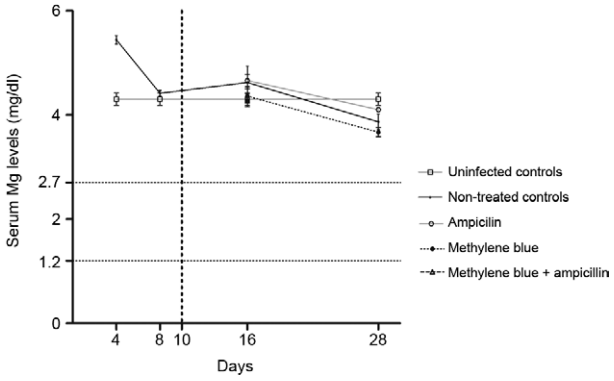

Fig. 2: serum levels of magnesium (Mg) in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mg/dL. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).

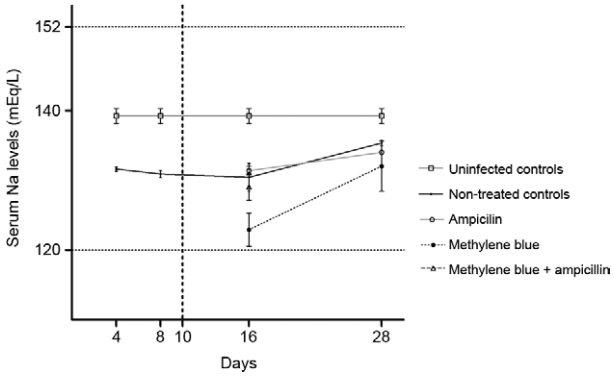

Fig. 3: serum levels of sodium (Na) in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mEq/L. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).

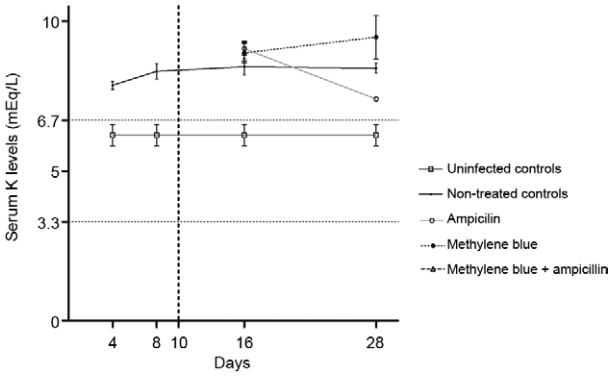

Fig. 1: serum levels of potassium (K) in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mEq/L. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).

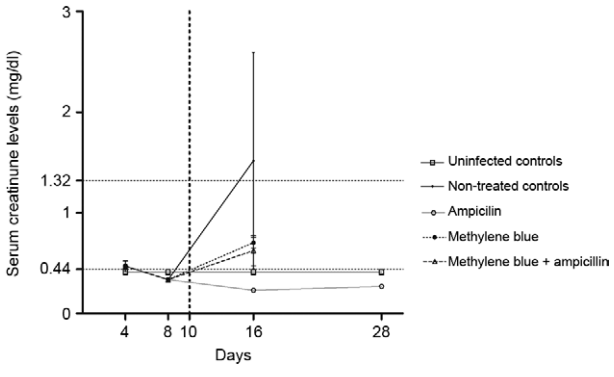

Fig. 4: serum levels of creatinine in the hamsters that were treated with ampicillin, methylene blue, both or no treatment. The vertical dashed line indicates the initiation of treatment. Values are expressed as mg/dl. The horizontal dashed lines indicate the previously reported reference values for laboratory hamsters (Tomson & Wardrop 1987).

nephritis was characterised as an infiltrate that extended to other renal parenchymal zones with one-two lesions per field of view at 100X magnification and grade +++ nephritis was characterised by lesions detected in more than two areas per field of view at 100X magnification. In the present study, the regeneration of the tubular epithelium was quantified using the criteria for foci of interstitial nephritis. Acute tubular damage (tubular cell swelling) was also estimated semi-quantitatively, as mild, moderate or severe.

Statistical analyses - Statistical analyses and graphical presentation of the data were performed using the Prism v4.03 software package (GraphPad Software Inc, La Jolla, CA USA). Numerical data were compared using the non-parametric Mann-Whitney *U* test when comparing two groups and using the non-parametric Kruskal-Wallis *U* test when comparing more than two groups. Survival curves were compared using the log-rank Mantel-Cox test. A p value < 0.05 was considered significant.

RESULTS

We selected the 10th day for the initiation of treatment, as this resulted in the survival rate closest to 50% (Table I). During the second and third preliminary experiments, in which the selected inoculum of 1,000 leptospires was used, ampicillin treatment initiated on day 10 resulted in survival rates of 33% and 40%, respectively.

The first experiment involved 80 hamsters. At both the day 4 and day 8 scheduled time points, 20 animals were euthanised and necropsied. Treatments were initiated on day 10. Another 20 hamsters were evaluated at the day 16 and day 28 time points, now assigned to four different treatment groups. Prior to day 16, deaths were observed in two untreated hamsters (both on day 11), four ampicillin-treated hamsters (on days 10, 10, 10 and 11) and five ampicillin + methylene blue-treated hamsters (on days 10, 10, 10, 11 and 13). At day 16, five animals were evaluated in each group. Then, prior to day 28, two additional animals from the methylene blue-treated group died (both on day 27). Thus, on day 28, all of the survivors were euthanised, which included three in the untreated group, three in the methylene blue only group and one in the ampicillin only group. No hamsters treated with both ampicillin and methylene blue survived until day 28. Blood and tissue samples were only collected from animals that survived until the scheduled time points.

At days 4 and 8, serum K levels were found to be significantly higher (Mann-Whitney *U*, $p < 0.05$) among the infected hamsters compared with uninfected hamsters. At day 16, all of the infected groups displayed higher serum K levels compared with the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the methylene blue and no-treatment groups still exhibited significantly higher levels of serum K compared with the controls. The dynamics of the serum K levels in the different treatment groups are shown in Fig. 1.

Both the uninfected and infected animals (regardless of the treatment group) showed higher serum Mg levels compared to historical (literature-based) reference values. For this reason, serum Mg measurements were repeated twice for each sample and samples from other uninfected hamsters collected after the end of experiment were found to show consistent serum Mg levels of 4.0-4+5 mg/dl. In all cases, these measurements confirmed similar results. Infected animals showed higher Mg levels compared to uninfected controls on day 4 and there was a trend toward decreasing levels of Mg detected on days 8, 16 and 28 among the infected hamsters. Serum Mg levels were significantly higher among the infected hamsters compared with the uninfected hamsters on day 4 (Mann-Whitney *U*, $p < 0.05$). None of the other differences were significant. The dynamics of the serum Mg levels in the different treatment groups are shown in Fig. 2.

Serum Na levels were within the normal range in all hamsters, though there was a trend toward lower Na levels among the infected animals compared with the uninfected controls. The serum Na levels were significantly lower among the infected hamsters compared with the uninfected hamsters on days 4 and 8. At day 16, all of the infected groups exhibited lower serum Na levels compared with the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the no-treatment group still presented significantly lower Na levels compared with the controls and the group receiving methylene blue exhibited a trend toward lower Na levels (Mann-Whitney *U*, $p = 0.06$). The dynamics of the serum Na levels in the different treatment groups are shown in Fig. 3.

The only difference observed between the groups of infected animals was that the methylene blue treatment group presented lower serum Na levels at day 16 compared with the other infected groups (Kruskal-Wallis, $p = 0.04$).

Serum creatinine levels peaked at day 16 and were higher in the untreated group. The serum creatinine levels in the untreated group were higher than normal reference limits and significantly higher than the levels in the hamsters in all of the treatment groups (Kruskal-Wallis, $p = 0.02$). Ampicillin administration prevented the elevation of serum creatinine at days 16 and 28. The dynamics of the serum creatinine levels in the different treatment groups are shown in Fig. 4.

The kidney samples from the hamsters euthanised at day 4 were uniformly normal when examined under light microscopy (data not shown). In contrast, the hamsters euthanised on day 8 presented the typical features of acute disease, such as diffuse massive tubular cell swelling with mild or no interstitial nephritis. Foci of tubular cell swelling were still detectable at days 16 and 28, but were not as large or as diffusely distributed as on day 8. Regenerative tubules and interstitial inflammation were detected to various degrees on days 16 and 28, but not on day 8. The frequency and severity of renal lesions did not differ in the infected hamsters on day 16 and 28. Illustrative images of the renal histopathology of these hamsters are displayed in Fig. 5.

A second experiment evaluated the outcome of infection when treatment was initiated immediately after the first death was observed among the infected animals (regardless of the assigned group). In this case, only 10% of the tested animals survived in the ampicillin group, whereas infection was uniformly lethal in the untreated, methyl-

ene blue-treated and methylene blue + ampicillin-treated groups (Table II). Additional methylene blue administration resulted in no improvement of outcomes, evaluated either in terms of survival or the period between infection and death (Log-rank Mantel-Cox test, p = 0.35).

DISCUSSION

The hamster model employed in this study did not reproduce the hypokalaemia that is commonly observed in human leptospirosis. The rapid and progressive development of hyperkalaemia most likely reflects experimental conditions in which supportive therapy is not feasible, such as venous rehydration. Even the surviving hamsters that were euthanised at day 28 presented elevated levels of serum K (Fig. 1). Antibiotic treatment and/or methylene blue treatment had no effect on serum K levels in the infected hamsters. Serum K levels were significantly higher among the infected hamsters compared with the uninfected hamsters on days 4 and 8. At day 16, all of the infected groups exhibited higher serum K levels than the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the methylene blue and no-treatment groups still exhibited significantly higher levels of serum K than the controls.

A similar pattern of Mg elevation was observed among the infected hamsters (Fig. 2). Although some Mg depletion might be expected based on clinical studies, rapid progression to severe renal failure could explain the retention of Mg. In contrast to the K dynamics, there was a trend toward decreasing levels of Mg observed on days 8, 16 and 28. The baseline serum Mg levels in the uninfected hamsters were the only measurements that were considerably different (higher) compared with the reference values for laboratory hamsters. The serum Mg levels were significantly higher among the infected hamsters compared with the uninfected hamsters at day 4. None of the other differences were significant.

High baseline serum Mg levels were not expected. Reference values for serum Mg (Fig. 2) in hamsters were obtained from a textbook (Tomson & Wardrop 1987), which were based in two previous studies using 164 and 19 hamsters (mean ± standard deviation of up to 2.5 ± 0.2 mg/dl in males from one study and 1.6 ± 0.4 mg/dl in females from the other). The range suggested from these data is indeed close to the reference serum Mg levels in humans. Another reference textbook indicated values with a considerably wider range, of 1.9-3.5 mg/dl (Gad 2007). However, a more recent textbook apparently ig-

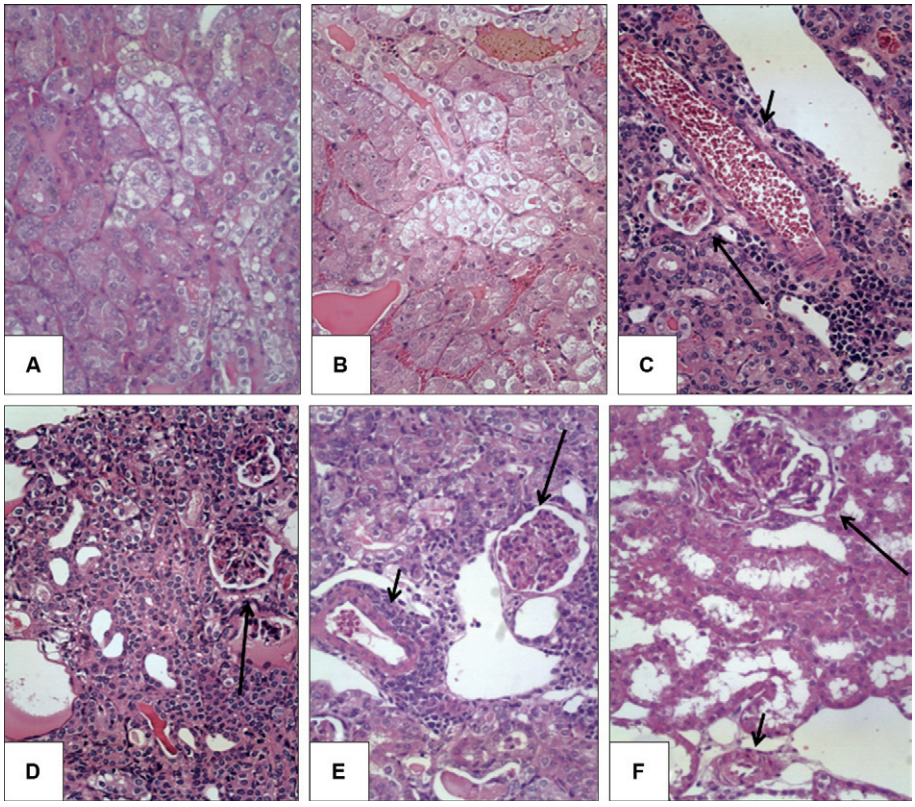

Fig. 5: typical lesions of leptospirosis in the infected hamsters. A: marked swelling of the epithelial cells of the proximal tubules in an untreated hamster at day 8; B: other focus of tubular cell swelling in an untreated hamster at day 16; C: mild interstitial nephritis with infiltrates rich in lymphocytes, plasma cells and macrophages surrounding an artery of an untreated hamster at day 16; D: regenerative changes of cortical tubular epithelium of an untreated hamster at day 16; E: mild interstitial nephritis with infiltrates rich in lymphocytes, plasma cells and macrophages surrounding the glomeruli and small arteries in the renal cortex of an ampicillin-treated hamster at day 28; F: normal kidney of an uninfected hamster at day 8. The short arrows indicate small arteries and the long arrows indicate the glomerulus. Haematoxylin-eosin (A-F) 200X.

nores these previous data and concludes that reference serum Mg values are not available for hamsters. Strikingly, the indicated reference values for other laboratory animals are as high as 2.0-5.4 mg/dl in rabbits, 3.5-4.1 mg/dl in guinea pigs and 3.6-4.0 mg/dl in chinchillas (Washington & van Hoosier 2012). In comparison to the normal range for guinea pigs, the baseline serum Mg levels found in the uninfected controls in the present study would considered be normal, while in comparison to the normal range in rabbits, the serum Mg levels found in all of our experimental groups would be within normal limits. For the purpose of the present study, the analysis was based on the comparison between all groups with the serum Mg levels detected in our uninfected controls. These values consistently ranged from 4.0-4.5 mg/dl in samples from the uninfected animals used in this study and from other uninfected hamsters in our laboratory. Although this is beyond the scope of our study, we speculate that reference values for serum Mg in hamsters should be reviewed or further evaluated based on the following observations: (i) the available data in the literature are scarce and show a wide range, (ii) the present results, both from animals evaluated during the study period and in different independent experiments afterwards, indicate considerably higher levels than have been previously reported and (iii) some related laboratory rodents are known to display considerably higher serum Mg levels.

There was a trend toward lower Na levels among the infected animals compared with the uninfected controls. However, all of the measurements were within the normal range of serum Na levels for hamsters (Fig. 3). The serum Na levels were significantly lower among the infected hamsters compared with the uninfected hamsters at days 4 and 8. On day 16, all of the infected groups exhibited lower serum Na levels compared with the uninfected animals, regardless of the treatment received. At day 28, the three remaining animals in the no-treatment group still presented significantly lower Na levels compared with the controls and the group receiving methylene blue exhibited a trend toward lower Na levels.

The methylene blue treatment group presented lower serum Na levels on day 16 compared with the other infected groups (Fig. 3). Any discussion regarding this finding is merely speculative, but it might be explained based on the combination of the following factors: (i) a syndrome of inappropriate antidiuretic hormone hypersecretion causes hyponatremia and is known to occur in some severe infectious diseases such as pneumonia and meningitis (Mendoza 1976) and (ii) local inhibition of NO production in the kidney interferes with Na reabsorption and may result in either depletion or retention (Manning & Hu 1994).

In this study, the dynamics of ionic changes were not found to be associated with any peculiar histopathologic features in the kidneys. The kidney samples from the hamsters that were euthanised at day 4 were uniformly normal when examined *via* microscopy (data not shown), despite the high serum levels of Mg detected in these animals (Fig. 2). The hamsters that were euthanised at day 8 presented the typical features of acute disease, such as diffuse and massive tubular cell swelling with mild or no interstitial nephritis. At this time point, marked hyperkalaemia was observed (Fig. 1); however, it was even higher on day 28, when the tubular changes were resolved and more severe manifestations of interstitial nephritis dominated the microscopic observations (Fig. 5E). In the present study, severe ionic changes persisted after recovery from the acute tubular changes. This last finding confirmed a previous clinical report, suggesting that tubular defects, such as impaired urinary concentration capacity, may last for months in patients (Daher Ede et al. 2004).

The hamsters that were euthanised on days 16 and 28 exhibited variable degrees of acute tubular changes, regeneration of tubular epithelia and interstitial nephritis. In a previous report on experimental leptospirosis in guinea pigs, de Arriaga et al. (1982) did not observe any association between the frequency or severity of interstitial nephritis and renal failure. In the same study, regenerative tubular changes were found to be associated with renal dysfunction, which may imply previous tubular necrosis. As shown in Fig. 4, serum creatinine levels peaked at day 16, when regenerative changes in the epithelial tubular cells were first detected. The serum creatinine levels in the untreated group were higher than normal reference limits and significantly higher than the levels in the hamsters from all of the treatment groups. In contrast, serum creatinine levels were not significantly different from other treatment groups. In this study, the measured serum creatinine levels were likely biased

TABLE II

Outcome of hamsters infected by *Leptospira interrogans* strain L1130 and treated with ampicillin, methylene blue, both or no treatment

Treatment	Survivors n/N (%)	Days to death
Untreated controls	0/11 (0)	7, 7, 8, 8, 9, 9, 10, 13, 14, 16, 16
Ampicillin	1/10 (10)	8, 10, 10, 10, 11, 11, 12, 14, 15
Methylene blue	0/10 (0)	7, 7, 9, 9, 9, 10, 10, 11, 11, 13
Ampicillin + methylene blue	0/9 (0)	8, 9, 9, 9, 10, 10, 10, 11, 15

n/N: number of survivors/total number of evaluated hamsters.

because serum samples were only collected from the hamsters that were euthanised at scheduled time points. Hamsters that died before the scheduled euthanasia date likely exhibited more severe ionic imbalances and renal dysfunction. Importantly, the histopathology was similar in all groups at day 16, even though the serum creatinine levels were higher in the untreated animals. We have previously demonstrated that late antibiotic treatment in this hamster model can prevent or reverse the loss of Na+/H+ exchanger 3 and NKCC2 expression in renal tubular cells (Spichler et al. 2007), which is a finding that may be linked to the effect of antimicrobial treatment on the renal dysfunction markers observed in this study.

As shown in Table I, deaths were not expected to occur in the treatment assay after 15 days of infection. Thus, we defined survivors as those animals that survived to the 16 and 28 day time points. There was no observable benefit of adding methylene blue to standard ampicillin treatment. We previously reported a similar lack of an effect for the antioxidant N-acetylcysteine as an adjuvant therapy (Spichler et al. 2007). However, the strict methodology applied in these studies is the best way to reproduce the clinical conditions related to late treatment of patients. Models of delayed experimental treatment avoid obtaining promising results associated with experimental treatments that may be initiated too early during experimental infection to yield reproducible benefits in clinical practice.

Additionally, we have previously reported that *inos*-knock-out C57Bl/6 mice display lower rates and severities of interstitial nephritis and that the absence of iNOS is not associated with increased bacterial dissemination in tissues (Bandeira et al. 2011). Prêtre et al. (2011) have reported that treatment with another NOS inhibitor, 4-aminopyridine (0.3 mg/kg daily, starting on the day of infection), without co-administration of antibiotics, results in an accelerated lethal outcome in hamsters and a higher mortality rate in C3H/HeJ mice as well as a higher leptospiral burden in their tissues. The design of this previous study was different from the one presented here, as the former did not test NOS inhibition as an adjuvant treatment in the late stage of acute infection. In contrast with the effects observed in other models of sepsis (Kirov et al. 2001, Kwok & Howes 2006, Heemskerk et al. 2008, Paciullo et al. 2010), methylene blue does not appear to be beneficial in this model of leptospirosis. Blocking NO production also had no effect on the ionic imbalance observed during acute leptospirosis in the hamster model.

One limitation of this study is the lack of information on the bacterial burden. While it is important to understand the dynamics of infection, the examination of leptospiral loads does not directly interfere with analyses of survival, ionic changes and the frequency of renal lesions. We have previously shown that ampicillin treatment is associated with the clearance of leptospiral antigens, which parallels the preservation of renal tubule transporter expression (Spichler et al. 2007). In addition, we have demonstrated that genetic deficiency of iNOS is not associated with differences in the leptospiral load in tissues from the C57BL/6 mouse model (Bandeira et al. 2011).

The present study also revealed that the hamster model is not practical for reproducing and therefore studying the common ionic disturbances (such as hypokalaemia and hypomagnesaemia) that are observed in patients with severe leptospirosis. These ionic changes may occur during experimental infection, but progress so quickly to typical acute renal failure that they could not be detected at the time points selected in the present study. Patients with leptospirosis usually receive aggressive supportive therapy (including fluid expansion) that blocks or retards progression to an oliguric/hyperkalaemic state. Such supportive therapy was not provided in this study, which may explain the rapid progression to a severe form of renal failure.

In our second experiment, a different strategy was employed to reproduce the late initiation of treatment of experimental leptospirosis. Infected hamsters were followed to detect clinical signs and treatment was initiated one day after the first death was observed. In this model, even antibiotic therapy had almost no effect on survival (only 1 out of 10 animals survived) and the addition of methylene blue to the treatment regime provided no improvement of outcomes (Table II). Notably, although antibiotics are highly recommended, even in late-stage leptospirosis in clinical settings, there is no evidence demonstrating positive effects on survival when antibiotic treatment is initiated in late-stage leptospirosis. Such studies will likely not be performed because of ethical concerns about denying patients antibiotic treatment, even if no clinical benefit has been demonstrated.

Hypokalaemia was not reproduced in our hamster model: all of the groups developed increased levels of serum K. Furthermore, in this model of acute infection, hypermagnesaemia (rather than Mg depletion) was observed. These findings may be associated with an accelerated progression to acute renal failure. Furthermore, adjuvant methylene blue treatment had no effect on serum Mg and K levels during acute-phase leptospirosis in hamsters. Late antibiotic treatment prevented the accelerated elevation of serum creatinine levels in this model.

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6 DISCUSSÃO

O tratamento padrão específico da leptospirose é o uso de penicilina sódica endovenosa durante 5 dias. Entretanto, a terapia antimicrobiana apresenta eficácia limitada se administrada após quatro dias do início dos sintomas (W.H.O., 2003). Desta forma, novas terapias adjuvantes são uma necessidade urgente para potencializar o efeito do tratamento antimicrobiano com objetivo de reduzir a letalidade da doença. Para isto, desenvolvemos um modelo experimental de início tardio da antibioticoterapia buscando mimetizar a situação clínica mais comum, na qual o paciente com leptospirose começa a ser tratado em média após quatro dias de sintomas. Neste modelo de tratamento tardio em hamsters, o início do tratamento adjuvante associado à terapia antimicrobiana padrão foi definido para dez dias após a infecção, pois, em dois de três experimentos o início do tratamento no décimo dia após a infecção com terapia antimicrobiana padrão foi o ponto que mais se aproximou da metade (50%) dos animais infectados mortos ao final do experimento. Desta forma, este ponto permitiria melhor avaliar o efeito de uma terapia adjuvante associado à antibioticoterapia sub ótima.

Os níveis séricos de potássio foram significativamente mais altos no grupo dos hamsters infectados nos dias 4 e 8 quando comparado com o grupo de animais controles não infectados. No dia 16 os grupos de animais infectados continuavam a apresentar níveis de potássio mais altos independente do tratamento recebido quando comparados com o grupo de animais controles não infectados. Já no dia 28, os níveis séricos de potássio mais altos foram apresentados pelo grupo de animais tratados apenas com azul de metileno e animais infectados sem nenhum tratamento em comparação com animais controles normais. Pacientes com leptospirose em geral apresentam insuficiência renal inicialmente não oligúrica e hipocalêmica (SEGURO *et al.*, 1990), que pode evoluir para grave desidratação, e perda de volume associada à necrose tubular aguda (COVIC *et al.*, 2003). Neste momento, sobrevém o quadro de oligúria e hipercalemia. Diversos trabalhos mostram que oligúria e hipercalemia são marcadores de mau prognóstico da leptospirose (DUPONT, H. *et al.*, 1997; DAHER, E. *et al.*, 1999; LOPES, A. A. *et al.*, 2001). Neste trabalho, os hamsters não reproduziram a hipocalemia observada na leptospirose humana. O

desenvolvimento rápido de hipercalemia pode refletir as condições experimentais em que o apoio de reidratação venosa não é possível.

Os níveis de magnésio foram significantemente mais altos nos hamsters infectados quando comparados com os hamsters não infectados no dia 4 (Mann-Whitney, $p < 0.05$). Esses níveis foram progressivamente diminuindo nos dias 8, 16 e 28 nos animais infectados. Neste estudo, não houve diferença na dinâmica de magnésio entre os grupos tratados. Os níveis séricos de magnésio dos animais controles não infectados e infectados independentemente do tipo de tratamento estavam acima dos valores de referência em comparação com a literatura. Além disso, as dosagens de magnésio foram repetidas duas vezes nas mesmas amostras. O resultado da segunda dosagem de magnésio, sobretudo em animais controles não infectados revelou de modo consistente níveis séricos de magnésio de 4,0-4,5 mg/dl. Os valores de referência de magnésio foram extraídos de um livro baseado em trabalhos anteriores, nos quais os níveis séricos de Mg para hamsters variaram entre a média de 2.5 ± 0.2 mg/dl em machos e 1.6 ± 0.4 mg/dl em fêmeas (TOMSON e WARDROP, 1987). Valores de referência com intervalos maiores (1.9 – 3.5 mg/dl) são indicados em outro livro de toxicologia em hamsters (GAD, 2007). Mais recentemente, outro livro sobre animais de laboratório concluiu que não existem valores de referência confiáveis para Mg sérico em hamsters. Já os valores de referência para outros animais de laboratório são elevados como 2.0-5.4 mg/dl em coelhos, 3.5-4.1 mg/dl em porcos da índia e 3.6-4.0 mg/dl em chinchila (WASHINGTON e VAN HOOSIER, 2012). Usando os valores de referência de cobaias, os níveis séricos de Mg de hamsters não infectados estariam normais. Usando os valores de referência de coelhos, todos as dosagens séricas de Mg, de todos os grupos do presente estudo, seriam normais. Mesmo que isto esteja fora do escopo do nosso estudo, concluímos que os valores séricos de magnésio precisam ser revistos, ou melhor avaliados já que os dados da literatura são escassos e pouco consistentes. Isto é importante no campo de pesquisa de leptospirose visto que hamsters são o modelo mais disponível para reprodução da doença aguda letal e que os distúrbios do magnésio em pacientes com leptospirose também requer maior atenção.

Neste estudo, as dosagens de sódio foram mais baixas nos animais infectados em comparação com os animais controles não infectados. Entretanto, todas as dosagens de sódio em todos os grupos estavam dentro dos valores de referência. Nos dias 4 e 8 os níveis séricos de sódio nos animais infectados foram significativamente mais baixos em comparação com animais controles não infectados e ainda no dia 16, todos os grupos continuavam a apresentar níveis séricos de sódio mais baixos independente do tratamento recebido em comparação com os animais controles não infectados. No dia 28 os animais infectados não tratados apresentaram níveis significativamente mais baixos de sódio em comparação com o grupo controle não infectados e os animais tratados com azul de metileno apresentaram níveis séricos ainda mais baixos de sódio. A única diferença observada entre os animais infectados foi apresentada pelo grupo tratados com azul de metileno que mostrou níveis séricos de sódio baixos no dia 16 quando comparado com outros grupos.

Estes achados podem ser explicados pela combinação de dois fatores: 1) Os baixos níveis de sódio em animais infectados pode ocorrer devido a síndrome de hipersecreção inapropriada do hormônio antidiurético. Este panorama causa a hiponatremia e ocorre em algumas doenças infecciosas graves como pneumonia e meningite (MENDOZA, 1976). 2) pela inibição local da produção do NO nos rins que pode interferir na reabsorção de sódio e consequentemente na depleção ou retenção deste íon (MANNING e HU, 1994).

Os resultados da dosagem de creatinina sérica mostraram que esse marcador elevou-se progressivamente com pico no dia 16. A elevação da creatinina sérica nos dias 16 e 18 foi prevenida com tratamento da ampicilina.

Hamsters foram eutanasiados e seus rins foram coletados no dia 4. As amostras de rim coletadas neste dia foram igualmente normais entre todos os grupos analisados. As amostras de rins coletadas no dia 8 apresentaram características típicas de doença aguda como tumefação de células tubulares, com padrão difuso e maciço, com pouca ou nenhuma nefrite intersticial. Já nos dias 16 e 28 foi possível detectar regeneração tubular e nefrite intersticial em vários graus. A frequência e gravidade das lesões renais não diferiram em hamsters infectados a partir do dia 16.

No segundo experimento, o tratamento foi iniciado imediatamente após o primeiro óbito, como modelo de tratamento tardio. Somente 10% dos animais tratados com ampicilina sobreviveram. Todos os animais avaliados dos demais grupos tiveram 100% de mortalidade. Nenhum efeito adicional do azul de metileno como terapia adjuvante associada à terapia antimicrobiana padrão foi observado.

7 CONCLUSÕES

- A hipocalemia e a hipomagnesemia observadas na leptospirose humana não foram reproduzidas em modelo hamster.
- O tratamento com ampicilina e, ou, azul de metileno não mostrou efeito benéfico sobre o magnésio e o potássio plasmáticos durante fase aguda da leptospirose em hamsters.
- O tratamento com antibiótico e, ou, azul de metileno impediu a elevação acelerada dos níveis de creatinina no soro deste modelo.
- A terapia adjuvante que combina azul de metileno com ampicilina não apresentou benefício na sobrevida de hamsters experimentalmente infectados.

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ANEXO 1 – ARTIGO PUBLICADO

Artigo publicado no periódico internacional Infection and Immunity

Attenuated nephritis in inducible nitric oxide synthase-knockout C57BL/6 mice and pulmonary haemorrhage in CB17 SCID and recombination activating gene 1-knockout C57BL/6 mice infected with *Leptospira interrogans.*

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Neste trabalho, Bandeira e colaboradores investigaram a associação entre o status imune e a frequência da hemorragia pulmonar; bem como a associação entre a presença do gene *iNOS* e a frequência da nefrite intersticial no modelo murino. Para avaliação da frequência da hemorragia pulmonar no modelo murino, os investigadores infectaram camundongos C57B/6 *rag1* KO, camundongos CB17 SCID e o seu controle correspondente camundongos (BALB/C selvagem) com *Leptospira interrogans* sorovar Copenhageni cepa Cop. Os resultados dessa investigação mostraram que todos os animais knock-out (C57B/6 *rag1* KO) e mutantes (CB17 SCID) morreram de leptospirose aguda enquanto os controles (BALB/C) sobreviveram sem sintomas de leptospirose. A frequência de hemorragia pulmonar nos animais *rag1* KO e SCID chegou a 94% e 100%, respectivamente. Estes achados sugerem que a hemorragia pulmonar não está relacionada a mecanismos autoimunes na leptospirose experimental. A investigação da frequência de nefrite intersticial no modelo murino foi realizada em camundongos C57BL/6 iNOS – KO (camundongos knock-out para o gene da Óxido Nítrico Sintase Induzível) e de seu controle correspondente: C57BL/6 selvagem. Todos os animais knock-out e controles selvagens sobreviveram sem sintomas de leptospirose. A quantificação de leptospiras nos rins, os títulos de anticorpos aglutinantes e anticorpos IgG específicos anti-*Leptospira* foram semelhantes em ambos os grupos. Entretanto, os camundongos C57BL/6 iNOS KO desenvolveram nefrite intersticial com menor frequência e com lesões mais leves quando comparado com seus respectivos controles.

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Attenuated Nephritis in Inducible Nitric Oxide Synthase Knockout C57BL/6 Mice and Pulmonary Hemorrhage in CB17 SCID and Recombination Activating Gene 1 Knockout C57BL/6 Mice Infected with *Leptospira interrogans*[▽]

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The aims of this study were to investigate the frequency of pulmonary hemorrhage (PH) in mice unable to produce functional B and T lymphocytes and to explore the effect of an inducible nitric oxide synthase gene (*Inos*) knockout (KO) on the frequency/severity of interstitial nephritis *in vivo*. We studied the outcome of infection by the virulent *Leptospira interrogans* serovar Copenhageni strain Cop. The animals used were *Inos* KO mice, recombination activating gene 1 (*Rag1*) KO mice, CB17 severe combined immunodeficiency (SCID) mice, and the respective wild-type (WT) C57BL/6 and BALB/c controls. The *Inos* KO and WT mice survived with no clinical symptoms of leptospirosis. The frequency and severity of nephritis was significantly lower in the *Inos* KO mice. All of the *Rag1* KO and SCID animals died of acute leptospirosis, whereas all of the WT mice survived. PH was observed in 57 and 94% of *Rag1* KO mice and in 83 and 100% of SCID mice, using inoculum doses of 10^7 and 10^6 leptospires, respectively. There was no evidence of PH in the WT controls. In conclusion, the loss of the *Inos* gene had a negligible effect on the outcome of leptospiral infection, although we observed a reduced susceptibility for interstitial nephritis in this group. Of note, the absence of functional B- and T-cell lymphocytes did not preclude the occurrence of PH. These data provide evidence that PH in leptospirosis may not be related only to autoimmune mechanisms.

Leptospirosis is a zoonosis with a wide clinical spectrum that includes fatal outcomes due to acute renal failure and pulmonary hemorrhage (PH). Pathogenic leptospires are carried by diverse mammalian reservoirs, and peridomiciliary rodents are the most important source of infection in urban settings (1). Major efforts in vaccine development and basic research on mechanisms of disease have been carried out in recent years; however, our knowledge of the genetic determinants involved in host protection and pathogenesis remains limited (10).

Among the diverse animal models used in leptospirosis research, guinea pigs and hamsters are the most suitable laboratory rodents for reproducing acute lethal infection (12, 16, 17). Rats are resistant to acute disease and are more suited to studies focusing on mechanisms of persistent infection (3, 13). The mouse model offers a broad array of immunological and genetic tools available for basic research; however, it has been poorly explored in leptospirosis. In previous reports, we described differences in the outcome of experimental leptospiral infection among distinct wild-type (WT) mouse strains (15) and the lack of significant effects on outcome of knockouts (KO) in the genes for tumor necrosis factor alpha receptor Rp55, gamma interferon, and interleukin 4 (2).

A potential role for autoimmunity in leptospirosis-associated PH was suggested based on observations in the guinea pig model of leptospirosis (12) and, to a lesser extent, in human patients with severe pulmonary hemorrhage syndrome (7). However, the involvement of auto-antibodies in PH was countered by a description of lethal PH in experimentally infected severe combined immunodeficiency (SCID) mice lacking functional B- and T-lymphocyte subsets (19). Rats are the prototype model of resistance to acute lethal infection (3), but consistent with the observation from SCID mice, rats treated with cyclophosphamide (which suppresses humoral immunity) develop PH (18). However, the observation of PH in SCID mice is not reliable because it was observed in the C3H/HeJ mouse strain background (19) but not the C3H background (14). In this study, we reproduced these experiments in the following murine models: CB17 SCID and C57BL/6 recombination activating gene 1 (*Rag1*) KO mice. CB17 SCID mice are unable to produce functional B and T lymphocytes due to a mutation in the *Prkdc* gene, which encodes a DNA-dependent protein kinase involved in DNA double-strand break repair and recombination. The strain is similar to the BALB/c strain except that it carries the *Igh-1b* allele from the C57BL/Ka strain. *Rag1* KO mice lack a gene that plays an important role in the rearrangement and recombination of the genes of immunoglobulin and T-lymphocyte receptor molecules during the process of VDJ recombination. Thus, *Rag1* KO mice are unable to generate specific B and T lymphocytes. Mutations in both *Prkdc* and *Rag-1* genes are listed as causes of human SCID. In

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TABLE 1. Evaluation of leptospirosis in iNOS-deficient mice infected with *L. interrogans* strain Cop

Mouse strain[a]	Expt	Size of inoculum	% of mice with nephritis[b] (no. with nephritis/total no.)		Leptospiral load [median (IQR)[c]]	MAT titer [median (IQR)]
			Positive	Severe		
Inos KO	1	10^3	57.1 (4/7)	28.6 (2/7)	10 (4.5)	400 (300)
WT			86.7 (13/15)	33.3 (5/15)	5 (7.5)	300 (200)
Inos KO	1	10^6	25.0 (2/8)	12.5 (1/8)	0 (2.5)	400 (350)
WT			73.3 (11/15)	40.0 (6/15)	3 (7.5)	400 (1,200)
Inos KO	2	10^6	33.3 (5/15)[d]	13.3 (2/15)	0 (4.0)	800 (800)
WT			80.0 (12/15)	40.0 (6/15)	3 (8.5)	800 (1,400)
Inos KO	3	10^6	40.0 (6/15)	20.0 (3/15)	4 (14)[d]	400 (600)
WT			66.7 (10/15)	26.7 (4/15)	1 (1.0)	400 (700)
Inos KO	Total	10^6	34.2 (13/38)[d]	15.8 (6/38)[d]	8.1 ± 17.9[e]	400 ± 600[e]
WT			73.3 (33/45)	35.6 (15/45)	7.6 ± 18.8[e]	400 ± 1,400[e]

[a] *Inos* KO, iNOS gene-deficient murine strain; WT, C57BL/6 wild-type control.
[b] Positive, grade + or higher nephritis; Severe, grade ++ or +++ nephritis.
[c] IQR, interquartile range.
[d] $P < 0.05$ compared to the control group.
[e] Median ± standard deviation.

our previous report, the C57BL/6 background mice exhibited high leptospiral loads in kidney samples and developed severe inflammatory lesions, while these features were not observed in the BALB/c mice (15).

We have also reported the association between high serum levels of nitric oxide (NO) and the severity of renal involvement in patients with severe leptospirosis (9) Renal production of NO could be involved in transport defects in renal tubular cells (4). *In vitro* studies have previously reported the activation of a broad range of inflammatory genes, such as those for transcription factor NF-κB, inducible nitric oxide synthase (iNOS), monocyte chemotactic protein-1, and tumor necrosis factor alpha, by renal tubular cells in response to exposure to leptospire-derived products. These findings have been interpreted as a molecular trigger for interstitial nephritis (20–22). The genetic deficiency of iNOS has not been investigated *in vivo*. Nitric oxide secreted during an immune response acts as a free radical and generates toxic products against bacteria. Thus, in theory, the genetic deficiency of iNOS could alternatively promote higher loads of leptospires in blood and tissues or result in less severe inflammatory lesions in kidneys.

The aims of this study are as follows: (i) to investigate the frequency of PH in mice unable to produce functional B and T lymphocytes in light of the hypothesis that PH in leptospirosis is related to immunopathogenesis/auto-antibodies and previous unreliable data on the frequency of this complication in SCID mice of the C3H and C3H/HeJ backgrounds and (ii) to explore the effect of iNOS gene (*Inos*) KO on the frequency and severity of interstitial nephritis *in vivo* in light of previous *in vitro* data suggesting that leptospiral products induce renal tubular cells to express proinflammatory genes, such as *Inos*.

MATERIALS AND METHODS

***Leptospira* strains and culture conditions.** *L. interrogans* serovar Copenhageni strain Cop was cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) modified Tween 80-bovine albumin medium (Difco Laboratories) at 29°C, and leptospires were counted in a Petroff-Hausser counting chamber (Fisher Scientific). This strain was passaged and reisolated from hamsters four times and stored at −70°C. Frozen aliquots were thawed and passaged in liquid medium 14 times prior to use as a low-passage-number isolate in the infection experiments. The virulence of this strain was evaluated in hamsters as described previously, and the 50% lethal dose (LD_{50}) was calculated to be ~164 leptospires (15).

Experimental murine model of leptospirosis. The murine strains used in this study were C57BL/6 *Inos* KO (B6.129P2-Nos), C57BL/6 *Rag1* KO [129S(Cg)-Rag1], CB17 SCID, and the respective C57BL/6 and BALB/c WT controls. All mouse strains were purchased from The Jackson Laboratory and maintained in the animal unit at Fiocruz-BA. Animals were monitored daily for clinical signs of disease (loss of activity, jaundice, external hemorrhage, and moribund state). The *Inos* KO and the WT control mice (7 to 15 per group) were inoculated by intraperitoneal injection (10^3 and 10^6 leptospires in 1 ml phosphate-buffered saline [PBS]) in one experiment. The following endpoints of infection were evaluated: survival, renal pathology, leptospiral load, and immune response (Table 1). The immunodeficient mice and the WT controls (5 to 15 per group) were inoculated by intraperitoneal injection (10^6 and 10^7 leptospires in 1 ml PBS) in one experiment. The following endpoints of infection were evaluated: survival, time between infection and death, and the frequency of gross pulmonary hemorrhage (Table 2). The Ethics Committee of the Oswaldo Cruz Foundation approved all animal protocols used in this study.

Gross pathology and light microscopy. Animals presenting a moribund state were euthanized immediately, and convalescent survivors were euthanized 28 days postinfection. Necropsies were performed immediately after euthanasia. At necropsy, lungs were examined to detect macroscopic PH. Only macroscopic hemorrhages were reported as PH for purposes of this analysis. In all cases, microscopic examination was performed to confirm the presence of massive alveolar hemorrhaging. One kidney was fixed in 4% formalin and embedded in paraffin, and 4- to 5-μm-thick sections were used for conventional histology. A semiquantitative estimation of interstitial nephritis was used as previously described (2). Briefly, in grade + nephritis, infiltrate was rich in macrophages and lymphocytes and restricted to periarterial areas; in grade ++ nephritis, infiltrate extended to other renal parenchymal zones with 1 to 2 lesions per field of view at ×100 magnification; and in grade +++ nephritis, lesions were detected in more than 2 areas per field of view at ×100 magnification. For purposes of this analysis, grades ++ and +++ were considered to be severe nephritis.

Imprint detection of leptospires. Imprints were obtained by direct pressure of the cut surface of the tissue sample onto poly-L-lysine-coated glass slides, and leptospires were visualized by immunofluorescence as described previously (5). The immunofluorescence-based leptospiral detection in imprint samples is the detection method of choice in our laboratory, as it has proved to be reliable and has the advantages of simplicity and reduced time to result compared to immunofluorescence in frozen sections. Importantly, while renal colonization may lead to crowding of leptospires in tubular lumens, imprint-based visualization easily identifies isolated leptospires and, thus, has the additional advantage of allowing

TABLE 2. Lethal outcome, days between infection and death, and frequency of macroscopic pulmonary hemorrhages in immunodeficient and immunocompetent mice of the correspondent background

Expt[a]	Size of inoculum	No. of deaths/ total no. of mice (%)	No. of days between infection and death [median (IQR)][b]	No. of mice with macroscopic PH/ total no. (%)
1	10^7			
Rag1 KO		7/7 (100)	9 (0.5)	4/7 (57)
B6		0/15	NA	0/15
CB17 SCID		12/12	7 (0)	10/12 (83)
BALB/c		0/15	NA	0/15
2	10^6			
Rag1 KO		5/5 (100)	11 (0)	5/5 (100)
B6		0/15	NA	0/15
CB17 SCID		15/15 (100)	10 (0)	15/15 (100)
BALB/c		0/10	NA	0/10
3	10^6			
Rag1 KO		12/12 (100)	9 (0)	11/12 (91)
B6		0/14	NA	0/14
CB17 SCID		15/15 (100)	10 (0)	15/15 (100)
BALB/c		0/10	NA	0/10
Total	10^6			
Rag1 KO		17/17 (100)	9 (2)	16/17 (94)
B6		0/29	NA	0/29
CB17 SCID		30/30 (100)	10 (0)	30/30 (100)
BALB/c		0/10	NA	0/10

[a] *Rag1* KO, recombination activating gene 1 knockout mice; B6, C57BL/6 strain; CB17 SCID, CB17 mice with severe combined immunodeficiency.
[b] IQR, interquartile range; NA, not applicable.

easier quantification. In a previous study, we used this assay to quantify leptospiral density in murine kidney samples (15). Leptospires were quantified in kidney imprints, and the results expressed as the mean value for 10 fields of view at ×400 magnification. Only easily identifiable, intact, spiral-shaped organisms were included. Imprint samples of lung and liver from animals that developed acute lethal disease were analyzed.

Serology assays. The microscopic agglutination test (MAT) was performed as described previously, except that only the *L. interrogans* serovar Copenhageni strain Cop was used as the live antigen (15). An in-house anti-*Leptospira* IgG enzyme-linked immunosorbent assay (ELISA) was performed as previously described (15).

Statistics. Statistical analyses and graphical presentation of the data were performed using the Prism version 4.03 software package (Graph Pad). Categorical data were compared by Fisher's exact test, and numerical data were compared by the nonparametric Mann-Whitney test; a P value of <0.05 was considered significant.

RESULTS

***Inos* gene-deficient murine model of leptospirosis.** Both *Inos* KO and WT mice, regardless of inoculum dose, survived with no clinical symptoms of leptospirosis. Furthermore, there were no significant differences between the reciprocal MAT titers for specific anti-*Leptospira* agglutinating antibodies or IgG antibodies in either group (Table 1 and Fig. 1). The data on renal pathology, the leptospiral load in kidney samples, and the MAT reciprocal titer are summarized in Table 1. Overall, the leptospiral load was slightly higher, but not significantly so, in kidney samples from the *Inos* KO mice than in kidney samples from the WT controls. Of note, in the third experiment (10^6 leptospires), a significantly higher leptospiral load was observed in the *Inos* KO group. The results from the three experiments at the 10^6 inoculum showed that the *Inos* KO mice were significantly less susceptible to interstitial nephritis (grade +) than the WT group (34 versus 73%, respectively; $P < 0.01$) and, particularly, were less susceptible to severe nephritis (16 versus 36%, respectively; $P < 0.001$).

B- and T-lymphocyte-deficient murine model of leptospirosis. Both the CB17 SCID and *Rag1* KO murine strains were highly susceptible to acute lethal leptospirosis (Table 2). The median interval from infection to death was 9 days in *Rag1* KO mice, regardless of the inoculum dose. The median intervals to death for infected CB17 SCID mice were 7 and 10 days for inoculum doses of 10^7 and 10^6 leptospires, respectively. All animals developed severe jaundice and presented typical target organ pathology, including acute tubular damage and detrabeculation of hepatocytes (Fig. 2). The infected WT controls survived until 28 days postinfection, with no symptoms of leptospirosis.

Overall, macroscopic PH was observed in 57 and 94% of *Rag1* KO mice infected with 10^7 and 10^6 leptospires, respectively. PH lesions were observed in 82 and 100% of the CB17 SCID mice infected with 10^7 and 10^6 leptospires, respectively. When PH was noted macroscopically, microscopic evaluation was used to confirm the presence of massive recent intraalveolar hemorrhaging (Fig. 2). The quantification of leptospires in the target organs of the immunodeficient mice found high loads of leptospires in all groups and experiments (Table 3). The leptospiral loads of the immunodeficient mice and WT controls were not compared because the immunodeficient mice died 7 to 10 days postinfection, while the WT mice survived and were only examined on day 28 postinfection. Thus, differences in leptospiral load could be attributed to the time point of infection (acute lethal disease versus convalescence) rather than the effect of immune status.

DISCUSSION

The loss of the *Inos* gene in mice had no apparent effect on their survival or development of agglutinating or specific IgG antibodies against *Leptospira*. In theory, impaired NO production during the immune response to leptospirosis could be

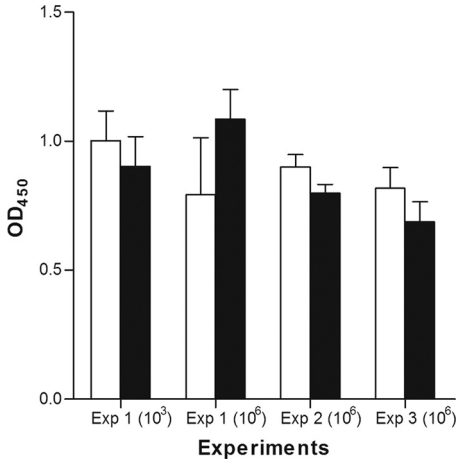

FIG. 1. ELISA analysis of serum anti-leptospiral IgG levels in infected mice 28 days postinfection. The graph compares antibody levels in C57BL/6 wild-type mice (solid bars) and inducible nitric oxide synthase knockout mice (open bars). The error bars represent the standard error of the mean for each group. OD_{450}, optical density at 450 nm.

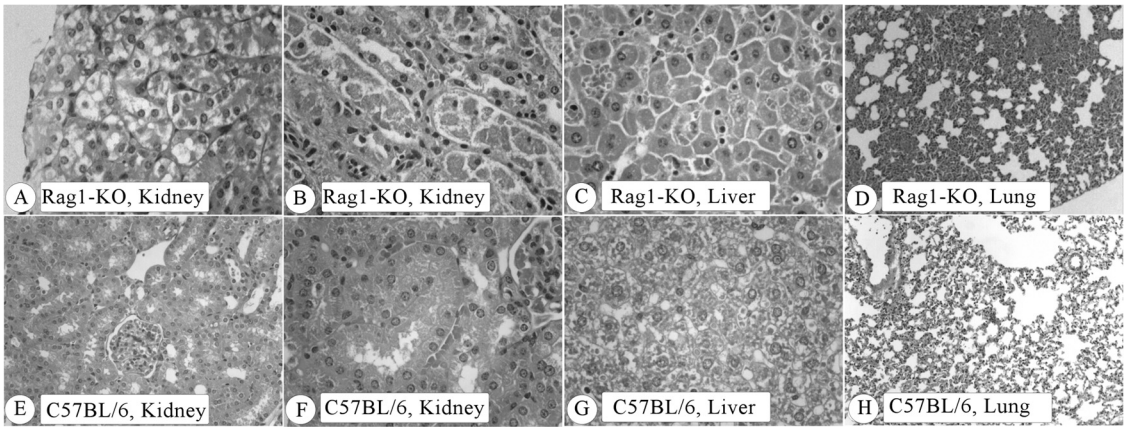

FIG. 2. Typical lesions of leptospirosis in a recombination activating gene 1 knockout C57BL/6 mouse that died 9 days after infection with strain Cop at a 10^6 inoculum. All tissue samples are stained with hematoxylin and eosin. (A) Marked cell swelling of epithelial cells of proximal tubules (×400). (B) Advanced necrosis of proximal tubules (×400). (C) Detrabeculation of hepatocytes (×400). (D) Microscopic foci of a pulmonary hemorrhage (×100). (E to H) For purposes of comparison, photomicrographs of tissue samples from a wild-type C57BL/6 mouse with no lesions are shown. (E and F) Kidney (×200 and ×400, respectively). (G) Liver (×400). (H) Lung (×100).

related to the slightly higher loads of leptospires observed in the tissue samples. However, the significantly higher leptospiral load observed was not reproducible (Table 1). The only significant difference observed between the *Inos* KO and the WT C57BL/6 mice was that the transgenic animals did not develop interstitial nephritis to the same degree or severity as the WT control. This result is in accordance with the hypothesis that the expression of proinflammatory markers by renal tubular cells *in vitro* after exposure to leptospiral products may be related to the development of interstitial nephritis *in vivo* (20–22).

The present study confirms previous reports, using mice of other backgrounds (C3H and C3H/HeJ), that immunodeficiency results in high susceptibility to acute infection, rapidly progressing to death (14, 19). In addition, immunodeficiency resulted in high loads of leptospires in the target organs, as seen upon necropsy, in accordance with a previous report on C3H/SCID mice (14). We observed the typical target organ pathology associated with leptospirosis, including acute tubular damage and detrabeculation of hepatocytes, similar to the pathology described in humans and in other models of lethal leptospirosis, such as hamsters (16).

TABLE 3. Quantification of leptospires in target organs of immunodeficient mice at necropsy

Mouse strain[a]	Expt	Size of inoculum	Median no. of leptospires (IQR[b]) in:		
			Kidney	Liver	Lung
Rag1 KO	1	10^7	154 (29)	60 (53)	49 (33)
CB17 SCID			26 (20)	20.5 (27.5)	2 (3.5)
Rag1 KO	2	10^6	220 (41)	258 (29)	159 (17)
CB17 SCID			141 (37)	181 (32)	155 (28.5)
Rag1 KO	3	10^6	29 (33)	24 (32)	29 (32.5)
CB17 SCID			160 (48)	143 (47.5)	123 (40.5)

[a] *Rag1* KO, recombination activating gene 1-deficient C57BL/6 strain; CB17 SCID, CB17 severe combined immunodeficiency strain.
[b] IQR, interquartile range.

There are insufficient data to attribute the pathogenesis of leptospirosis-related PH to a single mechanism. Furthermore, it is reasonable to assume that the severe pulmonary forms result from a multifactorial response to the direct toxic effects of exposure to leptospires, the effects of systemic inflammation on the alveolar wall, hemostatic disorders, and uremia (11). Nally and colleagues described a linear deposition of antibodies and complement in the guinea pig model, suggesting a potential role for autoantibodies in the pathogenesis of leptospirosis-associated PH (12). This mechanism associates leptospirosis-associated pulmonary disease with Goodpasture's syndrome, where autoantibodies against the glomerular basement membrane (GBM) cross-react with the alveolar septal matrix, causing massive alveolar hemorrhaging. However, the original evaluation of serum anti-GBM antibodies in leptospirosis patients with and without PH found no association between anti-GBM antibodies and lung disease. There was no difference in serum anti-GBM antibody levels between patient and control groups for either acute-phase or convalescent-phase sera (8). In addition, Craig and colleagues found no evidence for anti-GBM antibodies in 40 leptospirosis patients (6). In the present study, mice that were unable to produce functional B and T lymphocytes developed severe PH. This finding suggests that autoimmunity is not a major mechanism for PH in experimental leptospirosis, at least in the murine model and/or in *L. interrogans* serovar Copenhageni infections.

Conclusion. The absence of a functional *Inos* gene in the murine model had a minimal effect on the outcome of leptospiral infection, except for a significantly reduced susceptibility to the development of interstitial nephritis. The absence of functional B and T lymphocytes does not preclude the occurrence of PH. These data provide strong evidence that PH in leptospirosis is not related only to autoimmune mechanisms.

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ANEXO 2 – ARTIGO PUBLICADO

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Immunomodulatory treatment with thalidomide in experimental leptospirosis in Golden Syrian hamsters (Mesocricetus auratus)

Luciane Marieta Soares, Julio Oliveira Macedo, Everton Cruz de Azevedo, Cleiton Silva Santos, Marina de Queiroz Sampaio, Andreia Carvalho dos Santos, Mitermayer Galvão dos Reis and Daniel Abensur Athanazio. **Trans R Soc Trop Med Hyg** (2014)108 (2): 105-111.

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Neste trabalho avaliamos a talidomida como terapia adjuvante associada a antibioticaterapia e seus efeitos sobre a sobrevida, histopatologia e na quantidade de leptospiras nos tecidos de hamsters infectados experimentalmente. Hamsters foram infectados via intraperitoneal por *Leptospira interrogans* cepa L1-130, e foram separados em grupos: nenhum tratamento (NONE), talidomida (TAL), ampicilina (AMP) e ambos (AMP+TAL). A talidomida foi administrada via sonda orogástrica na dosagem de 50 mg/Kg diluída em óleo de linhaça (2ml/Kg) por três dias. Já a ampicilina por via intramuscular: 100mg/Kg/dia por seis dias. Foram realizados dois desenhos experimentais. Experimento 1: o tratamento foi iniciado 48h após o início dos sinais clínicos da doença. Experimento 2: o tratamento foi iniciado imediatamente após a detecção do primeiro óbito entre os animais infectados. Os resultados dessa investigação mostraram que no experimento 1 observou-se 100% de óbitos nos grupos animais controle sem nenhum tratamento (NONE) e tratados com talidomida somente (TAL). Não foi observado óbitos nos grupos animais tratados com ampicilina (AMP) e ampicilina + talidomida (AMP + TAL). A frequência de hemorragia pulmonar nos grupos NONE e TAL foi 100% e 63% respectivamente. Nenhum dos animais sobreviventes dos grupos AMP e AMP + TAL apresentaram hemorragia pulmonar macroscópica. No segundo experimento observou-se 100% de óbitos nos grupos NONE, TAL e 75% de óbitos nos grupos AMP e AMP + TAL. Hemorragia pulmonar macroscópica foi encontrada nos animais com doença aguda letal. No experimento 1, a diferença na contagem de leptospiras foi estatisticamente significante entre todos os grupos. Entretanto, essa diferença não foi observada entre os grupos NONE e TAL. No experimento 2, a contagem de leptospiras nos tecidos dos animais infectados foi significativamente mais baixa nos grupos que receberam tratamento, incluindo o grupo tratado com TAL. Em conclusão, a talidomida mostrou efeito limitado no modelo de leptospirose em hamsters.

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ORIGINAL ARTICLE

Immunomodulatory treatment with thalidomide in experimental leptospirosis in Golden Syrian hamsters *(Mesocricetus auratus)*

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Background: The benefit of antibiotics in leptospirosis is limited when treatment is started four days after symptoms appear, and new adjuvant therapeutic options are urgently needed.

Methods: Hamsters (*Mesocricetus auratus*) were infected by *Leptospira interrogans* strain L1-130, and groups were assigned based on no treatment (NONE), thalidomide only (TAL), ampicillin only (AMP) or both (AMP-TAL). Treatment was started two days after the onset of symptoms (experiment 1) and immediately after detection of the first death (experiment 2).

Results: Experiment 1: all hamsters from the groups AMP and AMP-TAL survived (n=8), while all hamsters from groups NONE (n=6) and TAL (n=8) died. The AMP and the AMP-TAL groups showed no renal or liver pathology and absent or very low leptospiral burden in target organs. Experiment 2: lethal outcome was observed in 6/6 hamsters in the NONE group, 8/8 in the TAL group, and 6/8 in both the AMP and AMP-TAL groups. Thalidomide showed no survival benefit when compared to hamsters treated with ampicillin alone. The TAL, AMP and AMP-TAL groups had very low tissue leptospiral counts.

Conclusion: Thalidomide had minimal impact on survival in the late treatment of leptospirosis hamster model.

Keywords: Animal disease models, *Leptospira*, Leptospirosis, *Mesocricetus auratus*, Thalidomide

Introduction

Leptospirosis is a widespread zoonosis caused by pathogenic leptospires. The disease occurs in distinct settings such as rural and/or occupational endemics, large urban areas with poor sanitation, or water sports and ecotourism related exposure.[1] Most human infections are asymptomatic or cause mild febrile illness that is indistinguishable from diseases caused by other infectious agents. However, 5–10% of human infections will evolve to severe forms, such as the Weil's triad of acute renal failure, hemorrhages and jaundice (with 5–30% related case fatality) and severe pulmonary hemorrhagic syndrome (with ≥50% related case fatality).[2–4]

Additional tools for the treatment of severe forms are urgently needed because a major issue in the management of patients is the limited effect of antibiotics when started more than four days after clinical onset.[4] The hamster model reproduces target organ pathology of human severe leptospirosis including jaundice, tubulointerstitial nephritis, liver cell disarray and pulmonary hemorrhages.[5] In experimental leptospirosis, ampicillin is the most popular and standardized antibiotic treatment used by different groups in therapeutic assays.[6–9] We have previously developed a hamster model of experimental leptospirosis to reproduce the late start of antibiotic (ampicillin) therapy and to test the adjuvant effect of the antioxidant N-acetylcysteine[7] and methylene blue—an inhibitor of soluble guanylyl cyclase—downstream the action of nitric oxide synthases[6] which had no additional benefit in experimental disease. Another potential target for adjuvant therapy in leptospirosis may be the cytokine cascade related to tumor necrosis factor alpha (TNF-α) because leptospirosis seems to share the association of high levels of pro-inflammatory markers of severity and lethality with some forms of sepsis, such as meningococcemia.[10–12] In addition, most data suggest leptospirosis and typical gram negative sepsis share similar pathogenesis with regard to systemic endothelial activation;[3] severe forms of both conditions share the same hemodynamic changes;[13] and acute respiratory distress syndrome is a common feature of both,[3] and a recent study suggested that severe pulmonary

hemorrhagic syndrome in hamsters is parallel with higher local expression of pro-inflammatory genes.[14]

Thalidomide is a well-known immunomodulatory drug that has been widely tested as a possible alternative for treatment of auto-immune diseases and cancer due to its inhibitory effects on TNF-α production and angiogenesis.[15] In particular, pre-treatment with thalidomide prolonged survival in the rat model of sepsis caused by multidrug-resistant *Pseudomonas aeruginosa*.[16] This is the only work which evaluates the effect of thalidomide on survival in an experimental model of sepsis. In a previous work, the same group showed that pre-treatment with thalidomide prevented TNF-α elevation in the bloodstream of rats in an experimental model of sepsis by *Escherichia coli*. In addition, thalidomide prevented secretion of TNF-α by human monocytes after exposure to *E. coli*.[17] In Brazil, thalidomide use is licensed, and the drug is distributed only through specific programs of the Ministry of Health to treat the following diseases: hanseniasis-related erythema nodosum, AIDS-related oral aphthous ulcers, multiple myeloma, systemic lupus erythematosus, and Graft-versus-host disease.[18,19]

In previous studies, we developed a model of late antimicrobial treatment in the hamster model of leptospirosis to test adjuvant therapies.[6,7] In this model, late start of ampicillin results in sub-optimal therapy with expected survival rates around 50%. This experimental setting tries to reproduce the common clinical practice in which patients are usually diagnosed late in the course of disease and antibiotics are started four or more days after onset of symptoms. Late antibiotic treatment is the major cause of persistent high case fatality rates in leptospirosis and, as a consequence, adjuvant therapies are urgently needed. The aim of this study was to test whether thalidomide affected survival and target organ pathology when used as an adjuvant therapy in the late introduction of antibiotic therapy hamster model of severe disease. We also investigated if immunomodulatory treatment with thalidomide affected leptospiral load in tissues.

Methods

Bacteria

Leptospires were cultivated in liquid Ellinghausen-McCullough-Johnson-Harris (EMJH) medium (Difco Laboratories, Detroit, MI, USA) at 29°C and counted in a Petroff-Hausser counting chamber (Fisher Scientific, Pittsburgh, PA, USA). A virulent clinical isolate from Brazil, the *Leptospira interrogans* serovar Copenhageni strain Fiocruz L1-130, was used in all experiments.[20] This strain was passaged four times in hamsters and stored at −70°C. The aliquots were thawed, and prior to use, they were passaged in liquid medium less than 15 times for a low-passage isolate in the infection experiments. The virulence of this strain was evaluated in hamsters as described previously, and the 50% lethal dose (LD50) was calculated to be 164 leptospires

Animals and study design

Nine-week-old female Golden Syrian hamsters (*Mesocricetus auratus)* (Fiocruz/BA), weighing 60–100 g, were used in all experiments. All hamsters were inoculated intraperitoneally with 10^6 leptospires in 1 ml EMJH medium. All animals were monitored daily for the presence of clinical signs, including alopecia, prostration and photophobia. Ampicillin was administered intramuscularly at a dose of 100 mg/kg/bid. Treatment started on the selected date and lasted for six days. Thalidomide was supplied as capsules for oral administration. Thalidomide was minced and diluted in commercial linseed oil and administered via a gastric tube at a dose of 50 mg/kg.[16] The respective dose of linseed oil administered was 2 ml/kg. Treatment started on the selected date and lasted for three days.

In the two following independent experiments, hamsters were assigned into the following groups: NONE (no intramuscular treatment and pure linseed oil, 2 ml/kg via a gastric tube); TAL (no intramuscular treatment and thalidomide, 50 mg/kg diluted in linseed oil, 2 ml/kg via a gastric tube); AMP (intramuscular ampicillin, 100 mg/kg/bid and pure linseed oil, 2 ml/kg via a gastric tube); and AMP-TAL (intramuscular ampicillin, 100 mg/kg/bid and thalidomide, 50 mg/kg diluted in linseed oil, 2 ml/kg via a gastric tube). In the first experiment, the start of the treatment was arbitrarily defined as 48 h after detection of clinical signs in most infected animals. Clinical signs included alopecia, prostration and photophobia. In the second experiment, as an alternative strategy to reproduce the late start of treatment schemes, treatments were started immediately after detection of the first death regardless of the assigned group.

Animals were euthanized in a carbon dioxide chamber when they appeared moribund or at the 28th day after infection (defined as survivors). Necropsies were performed immediately upon euthanasia, and kidney, lung and liver samples were fixed in 4% formalin, embedded in paraffin and cut into 4–5 μm sections for conventional histology.

Outcomes

The primary outcome was survival. Secondary outcomes that were investigated were: macroscopic pulmonary hemorrhages, tubulointerstitial nephritis, liver lesions and leptospiral load in tissues. At necropsy, the lungs were examined to detect macroscopic pulmonary hemorrhage (PH). Only macroscopic hemorrhages were reported as PH for the purposes of analysis. In all cases, microscopic examination was performed to confirm the presence of massive alveolar hemorrhaging.

Histology

One kidney of each animal was fixed in 4% formalin, embedded in paraffin and 4–5 μm thick sections were used for conventional histology. A semi-quantitative estimation of interstitial nephritis was used as previously described.[21] Briefly, grade + nephritis: infiltrate was rich in macrophages and lymphocytes were restricted to periarterial areas; grade ++ nephritis: infiltrate extended to other renal parenchymal zones with 1–2 lesions per field of view at 100× magnification; and grade +++ nephritis: lesions detected in more than 2 areas per field of view at 100× magnification. Acute tubular damage (tubular cell swelling), tubular regeneration, glomerular hemorrhage and loss of cohesion (liver-plate disarray) of liver cells were also assessed semi-quantitatively. To test whether immunomodulatory treatment with thalidomide affected leptospiral burden in target organs, we used immunofluorescence-based leptospiral detection and quantification in imprint samples of kidney, liver and lungs as previously described.[21–24] Leptospiral

load was expressed as the total count of spiral-shaped organisms in ten fields of view at 400× magnification.

Statistical analysis

Statistical analyses were performed using the GraphPad Prism 4.03 software package (Graph Pad, La Jolla, CA, USA). Categorical data were compared by Fisher's exact test, and numerical data were compared using a non-parametric Mann–Whitney test when analyzing differences between two groups. Categorical data were compared by Fisher's exact test, and numerical data were compared using a non-parametric Kruskal-Wallis test when analyzing differences between more than two groups.

Kaplan-Meier curves were established to compare the effects of thalidomide on survival. Curves were compared using log-rank (Mantel–Cox) test. A p value <0.05 was considered significant.

Results

In the first experiment, lethal outcome occurred in 6/6 and 8/8 of the NONE and TAL groups, respectively. No deaths were observed in animals assigned in the AMP and AMP-TAL groups. Median (25th to 75th percentile) days to death were 10 (10–10.8) and 11 (10.8–13.5) in the NONE and TAL groups, respectively. Survival curves are shown in Figure 1. The differences between curves were significant (log-rank Mantel–Cox test, $p<0.0001$). A direct comparison between curves of the NONE and TAL groups showed no difference ($p>0.05$).

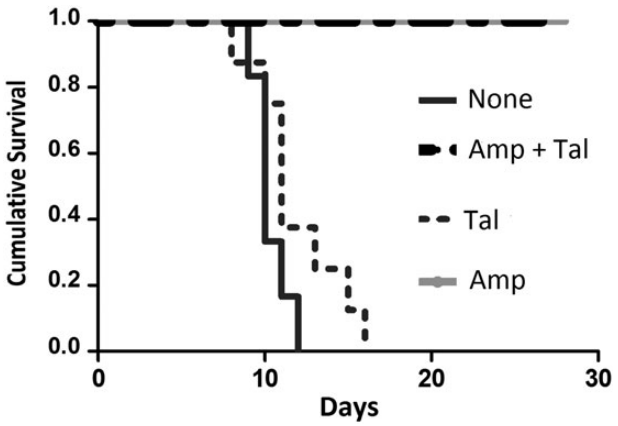

Figure 1. Survival curves of infected hamsters (10^6 *Leptospira interrogans* strain L1130) treated with ampicillin and/or thalidomide starting 48 h after the detection of clinical signs (experiment 1). Amp: ampicillin only; Amp-Tal: ampicillin and thalidomide; None: no treatment; Tal: thalidomide only.

The frequency of pulmonary hemorrhages detectable at gross examination was 6/6 (100%) and 5/8 (63%) in the NONE and TAL groups. The difference in the frequencies in both groups was significant (Fisher's exact test, $p< 0.05$). None of the animals defined as survivors (examined at necropsy on day 28) from the AMP and AMP-TAL groups showed macroscopic pulmonary hemorrhages. Table 1 also shows target organ pathology in kidneys and liver at the microscopic level. Animals dying of acute leptospirosis (NONE and TAL groups) showed acute tubular damage (tubular cell swelling), interstitial nephritis, regenerative tubular epithelium and glomerular hemorrhage, as well as liver plate disarray. These lesions were not detected in the AMP and AMP-TAL groups. Frequencies of acute tubular damage and liver plate disarray showed significant differences between groups ($p<0.05$), while frequencies of tubular regeneration, glomerular hemorrhages and interstitial nephritis were not different ($p>0.05$). In contrast, none of the lesions showed significant differences in the frequencies between the NONE and TAL groups. Images of target organ pathology, immunofluorescence-based leptospiral detection and quantification in imprint samples from experimental studies have been extensively illustrated in previous reports.[21–25]

We also investigated the leptospiral load in target organs to determine the extent that immunomodulation by thalidomide could affect the infection burden. Leptospires were detected in kidney samples of 6/6 (100%), 8/8 (100%), 1/8 (13%) and 0/8 hamsters from the NONE, TAL, AMP and AMP-TAL groups, respectively. The leptospiral count was 28 (3–881), 13 (2–131), 0 (0–0) and 0 (0–0) in the NONE, TAL, AMP and AMP-TAL groups, respectively. Significant differences were observed between groups but not between the NONE and TAL groups. In liver samples, leptospires were detected in 5/6 (83%), 2/8 (25%), 0/8 and 0/8 hamsters from the NONE, TAL, AMP and AMP-TAL groups, respectively. The leptospiral count was 13 (1–700), 0 (0–2), 0 (0–0) and 0 (0–0) in the NONE, TAL, AMP and AMP-TAL groups, respectively. Significant differences were observed between groups but not between the NONE and TAL groups. Leptospires were detected in the lung samples of 4/6 (67%), 5/8 (63%), 0/8 and 0/8 hamsters from the

Table 1. Target organ pathology in infected hamsters (10^6 *Leptospira interrogans* strain L1130) treated with ampicillin and/or thalidomide starting 48 h after the detection of clinical signs (experiment 1)

	Kidney n (%)				Liver n (%)	Lungs n (%)
	Tubular cell swelling	Interstitial nephritis	Tubular regeneration	Glomerular hemorrhage	Liver plate disarray	Macroscopy pulmonary hemorrhage
No treatment	4/5 (80)	0/5	1/5 (20)	3/5 (60)	4/5 (80)	3/5 (60)
Thalidomide only	4/7 (57)	1/7 (14)	2/7 (29)	3/7 (43)	3/7 (43)	4/7 (57)
Ampicillin only	0/8	0/8	0/8	0/8	0/8	0/8
Ampicillin and thalidomide	0/8	0/8	0/8	0/8	0/8	0/8

NONE, TAL, AMP and AMP-TAL groups, respectively. The leptospiral count was 6 (0–200), 1 (1–11), 0 (0–0) and 0 (0–0) in the NONE, TAL, AMP and AMP-TAL groups, respectively. Significant differences were observed between groups but not between the NONE and TAL groups.

In the second experiment, lethal outcome occurred in 6/6 (100%), 8/8 (100%), 6/8 (75%) and 6/8 (75%) of the NONE, TAL, AMP and AMP-TAL groups, respectively. The median (25th to 75th percentile) days to death was 6.5 (6–7.8), 9 (8–9.3), 9 (9–9.8) and 9.5 (8.3–10) for the NONE, TAL, AMP and AMP-TAL groups, respectively. Survival curves are shown in Figure 2. The differences between curves were significant (log-rank Mantel–Cox test, $p<0.0001$). A direct comparison between the curves of the NONE and TAL groups showed significant differences ($p<0.05$).

Pulmonary hemorrhages were detected in hamsters which succumbed to acute disease. The difference of frequencies between groups was not significant (Fisher's exact test, $p>0.05$). Table 2 also shows target organ pathology in kidneys and liver at the microscopic level. Frequencies of acute tubular damage and glomerular hemorrhages in animals that died of acute disease were not different between groups. The frequency of tubular regeneration was higher in the TAL, AMP and AMP-TAL groups, although the difference was not significant ($p>0.05$). Liver plate disarray was more common in the NONE group ($p<0.05$). Survivors exhibited no lesions in kidney and liver samples.

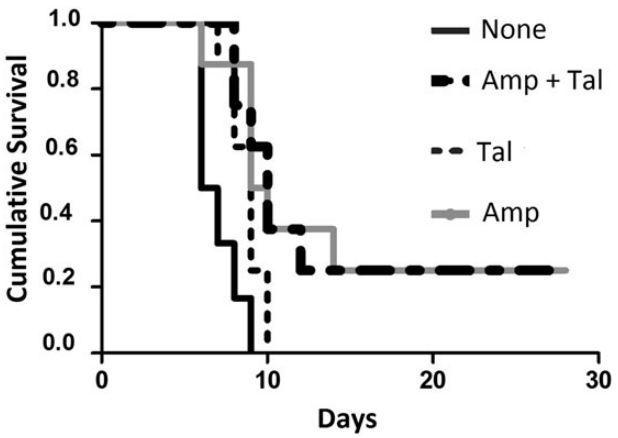

Figure 2. Survival curves of infected hamsters (10^6 *Leptospira interrogans* strain L1130) treated with ampicillin and/or thalidomide starting immediately after detection of the first death (experiment 2). Amp: ampicillin only; Amp-Tal: ampicillin and thalidomide; None: no treatment; Tal: thalidomide only.

In experiment 2, the leptospiral load was significantly lower in treated groups, including the TAL group. Leptospires were detected in kidney samples of 6/6 (100%), 4/8 (50%), 5/8 (63%) and 2/8 (25%) hamsters from the NONE, TAL, AMP and AMP-TAL groups, respectively. The leptospiral count was 234 (13–1250), 1 (0–5), 2 (0–20) and 0 (0–1) in the NONE, TAL, AMP and AMP-TAL groups, respectively. Differences were significant between the groups ($p<0.05$) and also between the NONE and TAL groups ($p<0.05$). Leptospires were detected in liver samples of 5/6 (83%), 2/8 (25%), 3/8 (38%) and 0/8 hamsters from the NONE, TAL, AMP and AMP-TAL groups, respectively. The leptospiral count was 14 (4–1213), 0 (0–1), 0 (0–2) and 0 (0–0) in the NONE, TAL, AMP and AMP-TAL groups, respectively. Differences were significant between groups ($p<0.05$) and also between the NONE and TAL groups ($p<0.05$). Leptospires were detected in lung samples of 4/6 (67%), 0/8, 1/8 (13%) and 0/8 hamsters from the NONE, TAL, AMP and AMP-TAL groups, respectively. The leptospiral count was 11 (0–311), 0 (0–0), 0 (0–0) and 0 (0–0) in the NONE, TAL, AMP and AMP-TAL groups, respectively. Differences were significant between the groups ($p<0.05$) and also between the NONE and TAL groups ($p<0.05$).

Discussion

Handling leptospires in vitro and dealing with experimental animal models of leptospirosis is a challenging endeavor. Minimal variations on in vitro passages, defrosting procedures and in animal conditions may result in significant changes in bacterial virulence and the outcome of experimental infections. In this context, it is difficult to establish a model for the late start of antibiotic therapy because more variables are added to this model, which is already known to show some inherent variability. While some innate difficulties of the late start model are clear, we believe that there is no other strategy to test urgently needed adjuvant therapies for leptospirosis under experimental conditions. The strategies presented herein aim to reproduce the common clinical setting in which patients with severe leptospirosis seek medical assistance after more than four days of symptoms: usually a

Table 2. Target organ pathology in infected hamsters (10^6 *Leptospira interrogans* strain L1130) treated with ampicillin and/or thalidomide starting immediately after detection of the first death (experiment 2)

	Kidney n (%)				Liver n (%)	Lungs n (%)
	Tubular cell swelling	Interstitial nephritis	Tubular regeneration	Glomerular hemorrhage	Liver plate disarray	Macroscopy pulmonary hemorrhage
No treatment	6/6 (100)	0/6	0	1/6 (17)	5/6 (83)	1/6 (17)
Thalidomide only	5/8 (63)	0/8	3/8 (38)	1/8 (13)	0/8	1/8 (13)
Ampicillin only	3/8 (38)	0/8	4/8 (50)	0/8	1/8 (13)	0/8
Ampicillin and thalidomide	4/8 (50)	0/8	3/8 (38)	0/8	1/8 (13)	2/8(25)

period of unspecific symptoms that can be easily confounded with dengue fever or a less severe self-resolving febrile illnesses.

The issue of immunomodulatory treatment in experimental leptospirosis has been poorly explored. Yukawa and colleagues tested the effects of combined or isolated therapies with penicillin and subcutaneous steroid therapy in gerbils.[26] The work did not reproduce the late start of treatment setting. Indeed, treatments were started three, two or one day before infection or at the moment of infection. As a consequence, this scheme reproduces chemoprevention rather than treatment of an established infection. In addition, as expected, gerbils pretreated with steroids had shortened survival. Similarly, in an experimental trial to compare ampicillin, ofloxacin, and doxycycline in hamsters, treatments were arbitrarily started after three days of infection, which was most likely too soon to reproduce clinical disease and the real setting of patients.[9] Even the work that suggested the benefit on survival of thalidomide in a sepsis model was also based on a pre-treatment with thalidomide thirty minutes before intraperitoneal infection with a multidrug-resistant blood isolate of *Pseudomonas aeruginosa*.[16] In that case, however, the disease had a short evolution, with deaths detected between 20 and 180 hours after infection. In the present study, we aimed to check differences on survival in a disease that usually follows a course of 10 to 14 days in lethal cases under experimental conditions. Therefore, the study design of a late start of antibiotic treatment and immunomodulation was imperative.

Five or six days of ampicillin are already standardized treatments for experimental leptospirosis. We arbitrarily defined the duration of thalidomide treatment as three days. As stated above, other studies focusing on immunomodulatory treatment with thalidomide or steroids were based in pre-infection administration of these. Thus, even arbitrarily defined, the study design of using thalidomide mirroring clinical practice is one of the strengths of the present work.

Regarding immunomodulation, we have previously tested N-acetylcysteine[7] and methylene blue[6] as potential adjuvants and observed no additional benefit in experimental disease when compared to ampicillin alone. In the former, study design included: treatments starting after detection of clinical signs of disease; and treatments starting six days after infection. In the latter, survival was evaluated in a experiment with the same design of the second experiment from this study: late treatment was reproduced by starting treatments immediately after detection of the first lethal outcome.[6]

It is noteworthy that even in the absence of supportive experimental data, some studies have already been performed on immunomodulation in patients with severe leptospirosis. There are anecdotal reports of clinical benefits of high doses of methyl prednisolone in patients with severe pulmonary hemorrhagic syndrome.[27–29] A report from Sri Lanka describes the experience after the introduction (in 2008) of bolus methylprednisolone as a standard therapy for severe leptospirosis: lethality dropped from 17/78 (22%) to 16/148 (11%).[30] However, a unique prospective randomized controlled trial on this issue was performed in Thailand: desmopressin or pulse dexamethasone were tested as adjuvant therapies in 68 patients with severe pulmonary hemorrhagic syndrome and showed no benefit.[31] In addition to their report of clinical benefit of steroid pulse therapy,[27] Trivedi and colleagues have reported that other immunosuppressive adjuvant treatments, namely cyclophosphamide and plasma exchange with immunosuppression followed by cyclophosphamide, improved survival.[32,33]

In experiment 1, the analysis of the effect of thalidomide on survival when used in combination with ampicillin therapy was not possible because all animals in the AMP and AMP-TAL groups survived. This was not expected based on preliminary data (Table 1); however, some variation in virulence of strains is common in practice of experimental leptospirosis. A minor loss of virulence in experiment 1 resulted, which indicated that starting treatment at the 10th day was too early to reproduce late therapy. Therefore, high survival (higher than expected) precluded analysis of the effect of combined ampicillin and thalidomide therapy. Although the difference was not significant, hamsters treated with thalidomide only survived longer than untreated animals. Thalidomide did not influence leptospiral load in tissues. Only animals that developed acute lethal disease showed pulmonary hemorrhages, kidney lesions and liver plate disarray. No pathology was detected in survivors.

Experiment 2 successfully reproduced late (suboptimal) ampicillin therapy. The rate of lethal outcome was the same (6/8, 75%) for both the AMP and AMP-TAL groups, and the survival curves were not significantly different. In contrast, hamsters treated with thalidomide only survived longer than untreated animals, and this difference was significant. Because lethal outcomes were observed in all groups, target organ lesions were detected in all groups. The frequency of two lesions, regeneration of tubular epithelium and liver plate disarray were significantly different between groups. More than from a direct effect of treatment, these differences may derive from a longer interval between infection and death. Liver plate disarray is a typical feature of acute-phase disease and may resolve in delayed cases of infection during renal disease progression. Conversely, regenerative epithelium most likely reflects previous acute tubular damage and may not be detected in cases of early lethal outcome. The fact that target organ lesions vary as a function of time (acute vs convalescence phase) is well characterized in humans and experimental leptospirosis.[5,34]

Comparing only the NONE group in experiments 1 and 2, the median was 10 (10–10.8) and 6.5 (6–7.8) (log-rank Mantel–Cox test, $p<0.0001$), respectively, thus implying that the strain, even if maintained at the same strict conditions, was more virulent in the second experiment. In experiment 1, symptoms were first detected at day 4. An overall median (25th to 75th percentile) day to death (regardless of assigned groups) was 11 (10–11.8). In experiment 2, symptoms were first detected at day 2, and the first death was observed at day 4. Therefore, treatment was started two days after the onset of symptoms. An overall median (25th to 75th percentile) day to death (regardless of assigned groups) was 9 (8–9.8). Treatments were started two days after appearance of clinical signs in both experiments, however, experiment 2 showed a more rapidly progressing disease. Thus, the variability of virulence and the different study design resulted in later treatment in experiment 2, allowing the occurrence of death in the ampicillin-treated groups. Comparing the outcomes of both experiments, it seems that waiting for the first death to begin therapy is a feasible way to reproduce the late start of treatment under these experimental conditions. Waiting for the first death to occur precludes the potential variability of the best day to begin therapy, as previously defined in other experiments as being due

to a possible loss of virulence of the strain, and avoids the requirement of time consuming preliminary experiments.

The leptospiral load in experiment 2 was consistently higher in the untreated groups when compared to the treated groups, including the TAL group. Differences in the leptospiral load in kidneys and lungs were significant between the NONE and TAL groups. An obvious conclusion is that short-term immunomodulation by thalidomide does not result in a higher leptospiral burden in tissues. However, this observation is not entirely unexpected. Thalidomide use may provide clinical benefit for patients with some infectious diseases, such as mycobacteriosis and microspordial diarrhea, in clinical patients; however, no direct effect on microorganism load has been demonstrated.[35,36] An alternative explanation, however, is that any treatment (including immunomodulation only) results in prolonged survival. Therefore, differences in bacterial load may reflect that the natural immune response is to clear leptospires from tissues over time, and thus, animals dying later may show a lower leptospiral concentration in tissues. Truccolo and colleagues have shown that after reaching the threshold of 10^4 leptospires/ml in blood, patients eventually succumb to leptospirosis despite aggressive antimicrobial treatment. Lethal outcome occurred even in patients who had reduced leptospiremia on follow-up: thus implying an immune mediated mechanisms associated with clinical complications.[37] As a result, acute leptospirosis may be fatal even if the leptospiral load decreases or is undetected in target organs.

In conclusion, we report a model of a late start of antibiotic therapy in the hamster model of leptospirosis to test adjuvant therapies. In the present study, thalidomide showed a limited effect on outcome, and thus, the present data does not support testing thalidomide as an adjuvant therapy in patients with leptospirosis. We hope that this report will encourage further research on potential adjuvant therapies for leptospirosis.

Authors' contributions: DAA conceived the study; DAA and LMS designed the study protocol; LMS, JOM, ECA, CSS, MQS and ACS carried out the experimental assessment; DAA and LMS drafted the manuscript; MGR critically revised the manuscript for intellectual content. All authors read and approved the final manuscript. LMS and DAA are guarantors of the paper.

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ANEXO 3 – ARTIGO PUBLICADO

Artigo publicado no periódico internacional Plos One

Detection and Quantification of Leptospira interrogans in Hamster and Rat Kidney Samples: Immunofluorescent Imprints versus Real-time PCR.

Adenizar Delgado Chagas-Junior, Caroline Luane Rabelo da Silva, Luciane Marieta Soares, Cleiton Silva Santos, Carlos Danilo Cardoso Matos Silva, Daniel Abensur Athanazio, Mitermayer Galvão dos Reis, Flávia Weykamp da Cruz McBride, Alan John Alexander McBride. **Plos One**, v. 7, p. e32712, 2012.

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Neste estudo, comparamos a imunofluorescência em imprint com o PCR em tempo real na quantificação de leptospiras em tecido renal de animais infectados experimentalmente com *Leptospira interrogans* cepa COP. Esta comparação foi realizada através da infecção de 12 ratos e 10 hamsters com 10^8 e 500 leptospiras respectivamente. A eutanásia foi realizada com a coleta de tecido renal para a realização do qPCR, imunofluorescência e cultura. A quantificação de leptospiras entre imunofluorescência e PCR em tempo real mostrou correlação positiva e significante. Apesar da alta sensibilidade da técnica de PCR em tempo real, o seu custo o torna inacessível a muitos laboratórios de pesquisa clínica e experimental. Por outro lado, a técnica de imunofluorescência em imprint apresenta custo baixo, tornando-o acessível a vários laboratórios de pesquisa clínica e experimental. Os dados deste estudo indicam que a detecção e quantificação de leptospiras através da técnica de imunofluorescência em imprint tem boa correlação com métodos de biologia molecular. Além disso, permite visualizar leptospiras íntegras, com sua morfologia preservada podendo ser aplicado em ensaios de imunidade esterilizante através de experimentos de vacina que avalia a presença de leptospiras nos rins do hospedeiro.

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PLoS one

Detection and Quantification of *Leptospira interrogans* in Hamster and Rat Kidney Samples: Immunofluorescent Imprints versus Real-time PCR

Adenizar D. Chagas-Junior[1], Caroline L. R. da Silva[1], Luciane Marieta Soares[2], Cleiton S. Santos[1], Carlos D. C. M. Silva[1], Daniel A. Athanazio[1,2], Mitermayer G. dos Reis[1], Flávia W. Cruz McBride[1], Alan J. A. McBride[1,3]*

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Abstract

A major limitation in the clinical management and experimental research of leptospirosis is the poor performance of the available methods for the direct detection of leptospires. In this study, we compared real-time PCR (qPCR), targeting the *lipL32* gene, with the immunofluorescent imprint method (IM) for the detection and quantification of leptospires in kidney samples from the rat and hamster experimental models of leptospirosis. Using a virulent strain of *Leptospira interrogans* serovar Copenhageni, a chronic infection was established in the rat model, which were euthanized 28 days post-infection, while the hamster model simulated an acute infection and the hamsters were euthanized eight days after inoculation. Leptospires in the kidney samples were detected using culture isolation, qPCR and the IM, and quantified using qPCR and the IM. In both the acute and chronic infection models, the correlation between quantification by qPCR and the IM was found to be positive and statistically significant ($P<0.05$). Therefore, this study demonstrates that the IM is a viable alternative for not only the detection but also the quantification of leptospires, particularly when the use of qPCR is not feasible.

Citation: Chagas-Junior AD, da Silva CLR, Soares LM, Santos CS, M. Silva CDC, et al. (2012) Detection and Quantification of *Leptospira interrogans* in Hamster and Rat Kidney Samples: Immunofluorescent Imprints versus Real-time PCR. PLoS ONE 7(2): e32712. doi:10.1371/journal.pone.0032712

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Introduction

Leptospirosis is an emerging neglected disease and is a major threat to public health, especially in developing and under-developed countries [1,2,3]. The global burden of leptospirosis has been estimated to be 500,000 cases per year [2,4], although this is probably under-estimated due to the lack of coordinated surveillance programs and poor diagnosis [5]. The gold standard method for the detection of pathogenic *Leptospira* spp. is culture isolation (CI), however it has poor sensitivity, is hampered by the slow growth of leptospires (requiring four to six months incubation [6]) and there is a high risk of culture contamination [7]. Direct detection by darkfield microscopy is even less sensitive and often results in false-positives due to misinterpretation [8]. The use of PCR, conventional or real-time (qPCR), for the detection of *Leptospira* spp. has resulted in major improvements in specificity and sensitivity [9]. Nevertheless, the widespread application of PCR for the detection of leptospires has been hampered by the risk of contamination with exogenous DNA and the associated risk of false-positives [10], plus reports of variable sensitivity [11].

Previous qPCR assays targeted genes common to all *Leptospira* spp., including *rrs* (16S rDNA) [12], *gyrB* [13], and *secY* [9] genes, or pathogen-specific genes including *lipL32* [14], *ligA* and *ligB* [15]. The *lipL32* gene, which encodes the immunodominant lipoprotein located in the leptospiral outer membrane, is highly conserved among the pathogenic serovars and is absent in the saprophytes [16,17]. These assays have been used to monitor renal colonization in experimental infection [15,18], to evaluate urinary shedding of leptospires in dogs [19] and for case confirmation in human subjects during outbreak investigations [20,21,22].

In the evaluation of vaccine candidates and leptospiral-host interactions, the detection and quantification of the leptospires is essential. qPCR has become the standard molecular tool for quantification purposes due to its high sensitivity [18]. However, not all laboratories have access to qPCR technology and the standard microbiological methods for quantification are not applicable to the pathogenic *Leptospira* spp. [7]. We previously developed an immunofluorescent imprint method (IM) for the direct detection of pathogenic *Leptospira* spp. by microscopy [23]. This technique is used routinely for detecting the presence of leptospires in the experimental models of leptospirosis used in our laboratories [24,25,26]. The aim of this study was to compare the IM with the standard method for quantification of leptospires, qPCR.

Methods

Ethics Statement

The Ethical Committee of the Oswaldo Cruz Foundation (Fiocruz) approved the animal protocols used in this study.

Leptospira strain and culture conditions

Leptospires were cultivated in liquid Ellinghausen–McCullough–Johnson–Harris (EMJH) medium (Becton Dickinson and Company, Franklin Lakes, NJ) at 29°C and counted in a Petroff–Hausser counting chamber. A highly virulent isolate from Brazil, *L. interrogans* serogroup Icterohaemorrhagiae serovar Copenhageni strain Cop, was used in all assays. The strain was passaged in hamsters four times and virulent isolates from kidney samples were cultured *in vitro* and stored at −70°C, as previously described [7]. Frozen aliquots were thawed and passaged in EMJH medium up to 14 times prior to use as a virulent isolate in the infection experiments. In previous experiments, the virulence of this strain was evaluated in hamsters and the LD_{50} was calculated to be ~164 leptospires [24].

Experimental models of leptospirosis

Laboratory animals (n = 23), the rat and hamster models of leptospirosis, were used in these experiments. Twelve, four-five week-old female Wistar rats (*Rattus norvegicus*, Fiocruz) were infected intraperitoneally with 10^8 leptospires and were euthanized 28 days post-infection (pi) as described previously [27]. Ten, nine week-old female golden Syrian hamsters (Fiocruz) were infected intraperitoneally with 500 leptospires ($3\times LD_{50}$) in 1 ml PBS, and euthanized 8 days pi. A hamster injected with PBS served as the negative control.

Collection of tissue samples and DNA extraction

Once euthanized, the abdominal cavity was opened and the kidneys were removed aseptically. Good laboratory practice was used in order to avoid DNA cross-contamination (including the use of a laminar airflow bench) and negative controls were included during all the DNA extraction procedures and qPCR steps. Total genomic DNA was extracted from approximately 25 mg tissue, using the QIAamp DNA Mini Kit (Qiagen, São Paulo, SP, Brazil). The tissue sample was a longitudinal section of the kidney that included the cortex and medulla regions, the same section was used in the IM method. The concentration of DNA obtained from tissues was determined with a spectrophotometer (NanoDrop ND 1000, NanoDrop products, Wilmington, DE).

Culture isolation of leptospires

CI was performed as previously described [27]. Briefly, whole kidney samples were homogenized in 5 ml EMJH, cell debris was allowed to settle for 10 min and 0.5 ml cleared homogenate was used to inoculate 5 ml EMJH. The cultures were incubated at 29°C and were examined regularly for growth, by darkfield microscopy, for up to 8 weeks.

Imprint detection

Imprints were produced by direct contact of the longitudinally cut surface of the kidney sample, the same region as used in the qPCR assay, onto a glass slide as described previously [23]. Briefly, the kidney imprints were dried, fixed in acetone for 3 min and incubated for 60 min with a primary rabbit polyclonal anti-leptospiral antibody at a dilution of 1:200. After washing in PBS, the imprints were incubated with a goat anti-rabbit IgG-FITC conjugate at a dilution of 1:500, washed in PBS and dried before visualization of stained organisms by fluorescence microscopy. Leptospires were quantified in imprint samples as the mean number of leptospires per 10 fields of view at a magnification of 1000×. Only intact spiral-shaped organisms were included in the calculation.

Real-time quantitative PCR

The *lipL32* gene was amplified using a previously described qPCR assay [19], with the following modifications. The qPCR reaction was performed using an Applied Bioscience 7500 thermocycler and the TaqMan Universal PCR Master Mix (Applied Biosystems, São Paulo, SP, Brazil). The standard curve was prepared from a *L. interrogans* serovar Copenhageni strain Cop culture (2×10^9 leptospires), centrifuged for 15 min at 10,000× g at 4°C. The recovered pellet was resuspended in PBS and washed by centrifugation (2×15 min, 10,000× g, 4°C). DNA was extracted from the pellet using a QIAamp DNA Mini Kit (Qiagen), as per the manufacturer's instructions. The concentration of the extracted DNA was calculated by spectrometry, optical density 260 and 280 nm (NanoDrop ND 1000), the standard curve was constructed by serial dilutions of the DNA stock. The samples were tested in duplicate, as was each dilution of the standard curve. Each run included a no-template negative control. Results were expressed as the number of genome equivalents per µg kidney DNA [18].

Statistical analysis

Statistical analyses were performed using the Prism v5 software package (GraphPad Software Inc., La Jolla, CA). The correlation between the methods was compared using the non-parametric Spearman's rank correlation (r_s), P values<0.05 were considered significant.

Results and Discussion

The end-point in the rat model of leptospirosis was a chronic non-lethal infection, as previously reported [27,28]. As expected, no deaths were observed, the animals were euthanized on day 28 pi and kidney samples were collected for evaluation by CI, IM and qPCR. In contrast, the hamsters developed an acute lethal leptospirosis and in previous reports we observed that symptoms/deaths due to leptospirosis typically occur from day 8 pi onwards [23,29]. Therefore, the hamsters were euthanized on day 8 pi and kidney samples were collected for evaluation of the presence and quantification of leptospires. All three methods were able to detect leptospires in the kidneys of all of the infected hamsters (10/10) and between 58 (7/12, qPCR) and 67% (8/12, CI and IM) of the infected rats (Table 1). Of note, two of the rats failed to establish a chronic infection. The uninfected controls were negative for the presence of leptospires.

Quantification of leptospiral load in the animal models was determined by qPCR, based on the assumption of one genome equivalent per spirochaete. The correlation coefficient of the standard curve was 0.999 and the efficiency was 92.4%, Fig. 1A. The limit of detection of the qPCR assay, based on serial dilutions

Table 1. Comparison of culture isolation (CI), the imprint method (IM) and real-time PCR (qPCR) for the detection of leptospires in animal models simulating chronic (rat) and acute (hamster) infection.

Animal model	Days post-infection	% Leptospire positive (No./total)		
		CI	IM	qPCR
Rat	28	66.6 (8/12)	66.6 (8/12)	58.3 (7/12)
Hamster	8	100 (10/10)	100 (10/10)	100 (10/10)

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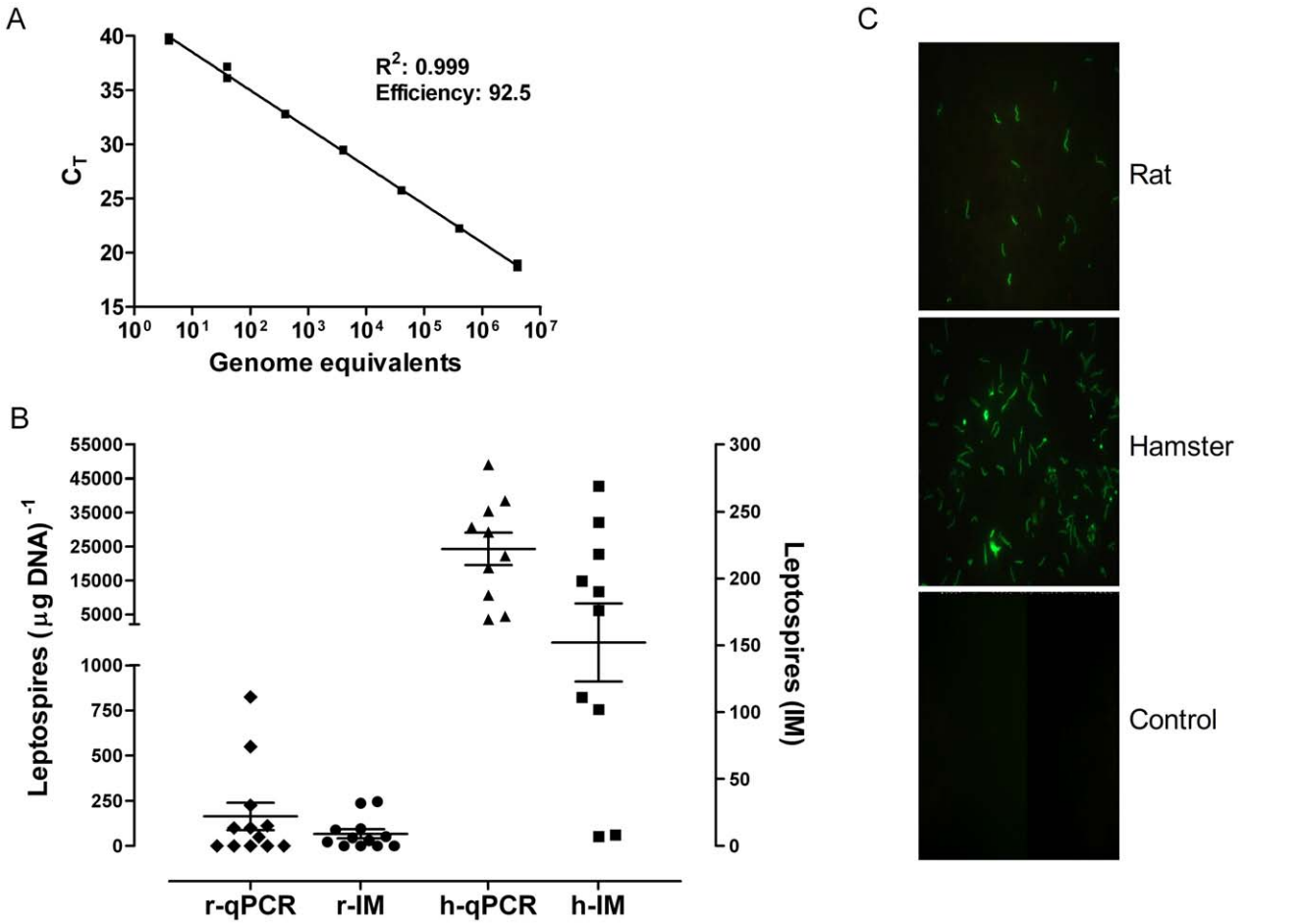

Figure 1. Quantification of leptospires by qPCR and the IM. A. Standard curve of the *lipL32* real-time PCR assay using DNA extracted from ten-fold serial dilutions of an *L. interrogans* strain Cop culture. Each DNA sample was quantified in duplicate and repeated twice. B. Quantification of the leptospiral load in the rat and hamster models. Rats were infected with 10^8 leptospires and were euthanized on day 28 pi. Hamsters were inoculated with 500 leptospires ($3\times LD_{50}$) and euthanized eight days pi. The leptospiral load in the kidneys was determined by qPCR (open symbols) and the IM (solid symbols). The leptospiral loads for the qPCR (leptospires per µg kidney DNA) and the IM (leptospires per 10 fields-of-view, ×1000 magnification) for the rat (r) and hamster (h) are presented as a scatter dot plot of the individual values for each animal, the horizontal line represents the mean value and the error bars the SEM. C. Representative examples of the imprint slides using kidney samples from an infected rat, a hamster and a non-infected control animal (magnification 1000×).
doi:10.1371/journal.pone.0032712.g001

of leptospiral genomic DNA, was estimated to be 4 genome equivalents per reaction or ca. 50 leptospires per µg DNA. This is similar to previous reports for the use of *lipL32* in a qPCR assay [19]. In the hamster model, the leptospiral load ranged from 3.6×10^3 to 4.9×10^4 (mean 2.4×10^4) leptospires per µg DNA and 7 to 269 (mean 138) leptospires in the IM. The qPCR and the IM exhibited a significant positive correlation ($r_s = 0.65$, $P = 0.02$), see Fig. 2. The leptospiral loads observed among the rats were lower, ranging from 50 to 825 (mean = 163) leptospires per µg DNA and 3 to 33 (mean = 9) leptospires for the qPCR and the IM, respectively. The correlation between the two methods was the highest observed $r_s = 0.70$, $P = 0.01$, Fig. 2. The correlation coefficients observed in hamsters and rats in this study indicated there was a moderate level of correlation between the methods.

O note, the leptospiral load in the rat model was lower than expected, with a mean of 163 leptospires per µg kidney DNA or a mean 9 leptospires per field-of-view, depending on the method used. Previously, we estimated the leptospiral load in rat kidneys (7–9 days pi) to be ca. 9 leptospires per field-of-view using immunofluorescent microscopy [27], similar to that seen in the current study using the IM. However, as the rat is the one of the main reservoir hosts for urban leptospirosis we expected a higher leptospiral load in the kidneys to allow for excretion to the environment and effective transmission of the disease [30]. A previous report found concentrations of up to 10^7 leptospires/ml urine 28 days p.i. [31]. A possible explanation is that the higher concentrations of leptospires are found in the renal tubules and not the surrounding kidney tissue in a chronic infection. The methodology used in the current study cannot determine the leptospiral load in renal tubules as the kidney sections used likely included only tubule cross-sections. Indeed, a limitation of the current study is that the concentration of leptospires in the urine of the infected rats was not determined.

The results reported in this study reinforce the usefulness of the IM for the detection of leptospires in commonly used experimental models of leptospirosis and confirm the results of the original imprint study [23]. Since its development, the IM has entered into routine use in our laboratories, in particular for evaluating the carriage status of animals used in the evaluation of potential vaccine candidates. A major drawback of the original study was

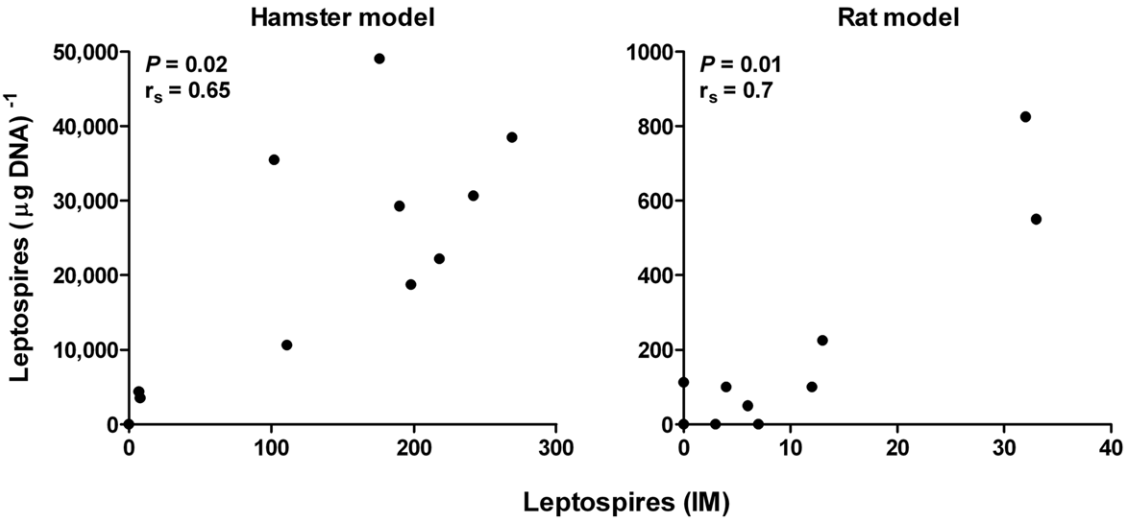

Figure 2. Correlation between the quantification of leptospires by qPCR and the IM. A significant ($P<0.05$), positive correlation was observed between the qPCR and the IM techniques in the experimental models of leptospirosis (rat and hamster) used in this study. The leptospiral loads for the qPCR are displayed as leptospires per µg DNA and in the IM as leptospires per 10 fields-of-view (×1000 magnification).
doi:10.1371/journal.pone.0032712.g002

the lack of a comparison with a qPCR assay to compare sensitivity of detection and quantification of the leptospiral load. This has been addressed in the current study. In terms of detection of leptospires (positive or negative), both the qPCR assay and the IM were comparable to the gold standard method, CI, in the hamster and rat models (Table 1). Note, a potential limitation of the IM and qPCR is their inability to distinguish between viable and non-viable leptospires and this is particularly relevant in determining sterilizing immunity conferred by vaccine candidates.

Another advantage of the IM is the ability to count the leptospires in the imprint samples. However, it was not known how the leptospiral count determined by the IM correlated with the absolute leptospiral load based on qPCR. Therefore, this study evaluated how the two methods covaried by an analysis of correlation in two animal models of leptospirosis. The values determined by qPCR and the IM were analysed for correlation and a significant, positive correlation was observed between the two methods in the hamster and rat models of leptospirosis (Fig. 2). The highest correlation was found in the rat model.

In conclusion, the results of the current study show that for the detection and quantification of leptospires the IM is equivalent to qPCR. In both acute and chronic infection models, the correlation between the IM and the qPCR methods was moderate. The imprint is a detection method that is cheap and is easily established in the laboratory. Furthermore, the fact that only intact leptospires are counted in the IM improves the probability that the observed leptospires are viable. Consequently, the IM is a valuable tool for use in evaluating secondary end-points, such as sterilizing immunity, during vaccine candidate trials and in determining the presence of leptospires in clinical samples.

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Author Contributions

Conceived and designed the experiments: ADCJ AJAM. Performed the experiments: ADCJ CLRS LMS CSS CDCMS FWCM. Analyzed the data: ADCJ AJAM. Contributed reagents/materials/analysis tools: DAA MGR FWCM. Wrote the paper: ADCJ DAA MGR FWCM AJAM.

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CONCLUSÕES DO PROJETO DE TESE E PESQUISAS RELACIONADAS

- A terapia adjuvante com azul de metileno não mostrou efeito benéfico sobre o Mg e K plasmáticos durante a fase aguda da doença e na sobrevida do modelo hamster experimental de leptospirose.
- A ausência do gene funcional *Inos* - KO no modelo murino reduziu o desenvolvimento de nefrite intersticial.
- A ausência funcional de linfócitos B e T não impede a ocorrência de hemorragia pulmonar no modelo murinho.
- A talidomida mostrou efeito limitado como terapia adjuvante associada a antibioticoterapia e desta forma não favorece o uso desta droga em pacientes com leptospirose.
- Em ambos modelos de infecção aguda e crônica, a correlação de detecção de leptospiras entre IM e qPCR foi moderada.