UNIVERSIDADE ESTADUAL PAULISTA

FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA

EFICÁCIA TERAPÊUTICA DE RNAS DE INTERFERÊNCIA (siRNAS) E AVALIAÇÃO DA RESPOSTA IMUNE EM CAMUNDONGOS INFECTADOS COM VÍRUS DA RAIVA DE ORIGEM DE CÃO E DE MORCEGO

CAMILA MICHELE APPOLINÁRIO-HARARY

Botucatu – SP

Junho/2014

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CAMILA MICHELE APPOLINÁRIO-HARARY

Tese apresentada junto ao Programa de Pós-Graduação em Medicina Veterinária para obtenção do título de Doutor.

Orientadora: Prof. Dra.Jane Megid

Título: EFICÁCIA TERAPÊUTICA DE RNAS DE INTERFERÊNCIA (siRNAS) E AVALIAÇÃO DA RESPOSTA IMUNE EM CAMUNDONGOS INFECTADOS COM VÍRUS DA RAIVA DE ORIGEM DE CÃO E DE MORCEGO

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DEDICATÓRIA

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"Não há lugar para a sabedoria, onde não há paciência "

(Santo Agostinho)

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RESUMO

A raiva é uma doença infecciosa letal que mata mais de 55 mil pessoas por ano em todo o mundo, embora a morte possa ser evitada se um tratamento pós-expositivo baseado no uso de vacina anti-rábica e imunoglobulinas for aplicado a tempo. Após o aparecimento dos sinais clínicos, não existe qualquer terapia eficaz disponível. As citocinas e quimiocinas são cruciais no desenvolvimento da resposta imune do hospedeiro. Este estudo teve como objetivo avaliar a expressão gênica de citocinas e quimiocinas relacionadas à resposta imune e também avaliar a eficácia da terapia com siRNAs em camundongos inoculados com virus de cão ou de morcego. Os resultados demonstraram que o perfil de expressão de citocinas e quimiocinas foi intrínseco `a variante viral e a produção precoce destas sugere ser mais importante do que seus níveis de expressão para a sobrevivência na raiva. Em relação à avaliação da terapia com siRNAs, embora nenhuma diferença tenha sido observada na taxa de letalidade entre os grupos tratados e não-tratados, a avaliação clínica de animais inoculados com a variante de cão mostrou menor severidade da doença clínica no grupo tratado quando comparado ao seu controle, associado a uma baixa expressão do gene N e de todos os marcadores imunológicos avaliados aos 5 dias. Os resultados deste estudo forneceram alguma evidência da eficácia da terapia com siRNA em infecções causadas pela variante de cão, a despeito da causada pela variante de morcego.

Palavras chave: Raiva; Resposta imune; siRNA; Cão; Morcego

APPOLINARIO-HARARY, C.M. **Therapeutic efficacy of interfering RNAs (siRNAs) and immune response evaluation in rabies infected mice due to dog virus and bat virus.** Botucatu, 2011. 131 p. Tese (Doutorado) – Faculdade de Medicina Veterinária e Zootecnia, Campus de Botucatu, Universidade Estadual Paulista.

ABSTRACT

Rabies is a lethal infectious disease that kills more than 55 thousand persons per year worldwide although death can be avoided if a post-exposure prophylaxis based in anti-rabies vaccine and immune globulins can be applied on time. After the onset of clinical signs, there is no effective therapy available. Cytokines and chemokines are crucial for host immune response development. This study had as purpose to evaluate the gene expression of cytokines and chemokynes related to the immune response and also to evaluate the efficacy of siRNAs therapy in mice inoculated with dog or bat virus. Results demonstrated that the gene expression profile was intrinsic to virus variant and the precocious production seemed to be more important than their expression levels for rabies survival. Therapy with siRNAs therapy showed no difference in the lethality rate between treated groups and controls but clinical evaluation of animals inoculated with dog variant showed less severity of clinical disease in the treated group compared with control, also associated with a low expression of *N* gene and of all immune markers evaluated at 5 days. Results provided some evidence of the efficacy of siRNAs therapy in infections due to dog variant but not due to bat variant in the present study.

Key words: Rabies; Immune response; siRNA; Dog; Bat

CAPÍTULO 1

Introdução

A Raiva é uma zoonose relatada em todos os continentes, fazendo-se exceção a Antártida. Causa aproximadamente 55-60 mil mortes ao ano, principalmente nos países asiáticos e africanos (WUNNER e BRIGGS, 2010; WHO, 2013). O vírus da raiva (RABV) causa encefalite aguda e, após o início dos sinais clínicos, a letalidade é de 100% na maioria dos casos, sendo considerada a maior taxa de caso-fatalidade dentre as enfermidades infecciosas (FOOKS et al., 2014). No entanto, pode ser evitada por meio de terapia pós-expositiva, baseada no uso de vacina anti-rábica e soro hiper imune, aplicadas de maneira adequada e em tempo hábil (RUPPRECHT, WILLOUGHBY e SLATE, 2006). Apenas dez sobreviventes de um quadro clinico de raiva estão relatados na literatura científica, embora apenas oito possam ser considerados pelo correto diagnóstico (JACKSON, 2013). Um fator comum a todos eles é a presença precoce de anticorpos anti-rábicos soroneutralizantes no líquido cefalorraquidiano, o que não acontece nos casos de óbito, quando estes anticorpos somente são detectados numa fase terminal, indicando que uma pronta resposta imune no sistema nervoso central (SNC) desempenha um papel importante no desfecho clinico (JACKSON, 2014). No entanto, a ativação da resposta nos casos de raiva, não esta limitada apenas à capacidade do hospedeiro em lidar com o agente agressor, mas diretamente relacionada `a patogenicidade da amostra viral, uma vez que, estudos demonstram que quanto mais patogênico o virus, maior a capacidade dele em evadir a resposta imune celular e gerar pouca inflamação no SNC (BALOUL e LAFON, 2003; WANG et al., 2005; LAOTHAMATAS et al., 2008). Durante a infecção, a resposta imune imediata envolve a liberação de diversas citocinas e quimiocinas, tanto pelos neurônios lesados como pelas células da glia, no caso das afecções que acometem o SNC (GRIFFIN, 2003). Estas citocinas e quimiocinas são responsáveis por conter a progressão do virus até que uma resposta imune celular especifica, com consequente produção de anticorpos seja instaurada, sendo a inibição destes mediadores, um

mecanismo de evasão do virus da raiva (CHOPY et al., 2011; NIU et al., 2011). Acredita-se também que as amostras de morcego tendem a ser menos patogênicas do que as amostras oriundas de cão, uma vez que nos dois casos de completa recuperação dos pacientes acometidos por raiva, uma jovem americana e um jovem brasileiro, o animal agressor havia sido um morcego (LAFON, 2005; JACKSON, 2014)

O conhecimento dos mecanismos de replicação e patogenicidade viral que possuímos na atualidade, ainda não foram suficientes para a elaboração de uma terapia comprovadamente eficaz contra RABV, sendo novas drogas e possíveis terapias sempre consideradas pelos pesquisadores da área, como é o caso da terapia utilizando o mecanismo de interferência pelo RNA, também conhecida por RNAi. Os RNAs de interferência vem sendo testados no tratamento de vários processos infecciosos, com resultados promissores, inclusive nas infecções pelo virus da raiva *"in vitro"* e "*in vivo*" (BRANDÃO et al., 2007; GUPTA et al., 2012; YANG et al., 2012; MESHRAM, 2013) no entanto, poucos utilizam amostras de rua do virus nos estudos experimentais, o que dificulta a avaliação da real eficácia desta terapia.

O estudo apresentado a seguir, teve como objetivos, avaliar a eficácia terapêutica de RNAi no tratamento da raiva experimental em camundongos infectados com amostras de rua do virus da raiva, sendo uma de cão (variante 2) e outra de morcego hematófago (variante 3); além de avaliar, no cérebro dos animais, a resposta imune induzida por estas distintas variantes de RABV.

Revisão de Literatura

Raiva: aspectos gerais e imunológicos.

O vírus da raiva (RABV), pertence ao gênero *Lyssavirus*, família *Rhabdoviridae* e ordem *Mononegavirales* (FAUQUET et al., 2005). É composto por um genoma de 12 kb, que sintetiza cinco

proteínas, sendo elas nucleoproteína (N), fosfoproteína (P), matriz (M), glicoproteina (G) e RNA-dependente-RNA polimerase (L) (JOHNSON at al., 2010). Possui caráter neurotrópico e causa encefalite aguda e fatal nos mamíferos, sendo as ordens *Carnivora* e *Chiroptera* as principais responsáveis por perpetuarem o vírus na natureza (RUPPRECHT et al., 2002).

RABV é transmitido, na grande maioria dos casos, pela saliva de um animal infectado por meio de mordidas, arranhões e contato com mucosas. Invade o Sistema Nervoso Central (SNC) por meio da ligação com receptores neuronais, presentes nas junções neuromusculares dos neurônios motores ou nas terminações dos neurônios sensoriais. Vários receptores podem ser utilizados, como o da acetilcolina, molécula de adesão da célula neuronal ou o receptor do fator de crescimento neuronal (WANG et al., 2005). A partir desta ligação, o virus é transportado até o gânglio dorsal da medula espinhal, onde sofre uma primeira replicação que permite sua detecção inicial. Através do transporte axonal, RABV chega ao cérebro, sendo este o local de eleição para uma replicação intensa, realizando em seguida uma migração centrifuga, que permite com que o virus se dissemine pelo corpo sempre por meio das terminações nervosas, fato este, que explica a possibilidade de transmissão da raiva nos casos de transplante de órgãos (VORA et al., 2013), incluindo as das glândulas salivares, por onde ele é excretado, completando seu ciclo ao infectar um novo hospedeiro (JOHNSON et al., 2010), O período de incubação da doença em seres humanos pode ser de semanas, meses e mais raramente, de anos, sendo que neste último caso, acredita-se que o vírus se mantenha em estado inativo ou replica-se em níveis muito baixos no tecido muscular próximo ao local de entrada. Após a entrada no SNC, sinais e sintomas ocasionados pela infecção, como dor/parestesia no local da agressão, febre, fadiga, fraqueza dos membros, dores de cabeça, confusão mental e ansiedade são relatados e precedem o quadro clinico de encefalite (JOHNSON et al., 2010).

A replicação viral ocorre no corpo celular dos neurônios, onde as proteínas virais podem ser detectadas nos dendritos, local onde se concentra o retículo endoplasmático rugoso, favorecendo a produção proteica. A propagação do vírus ocorre através de transferência transneuronal, por meio de vesículas axonais que são liberadas nas sinapses. Para que esta propagação seja bem sucedida, é necessário que os axônios e dendritos dos neurônios sejam preservados até que todo o processo de produção, montagem e liberação das novas partículas virais esteja completo (UGOLINI, 2010; KLINGEN et al., 2008; GUIGONI E COULON, 2002). No período mais tardio da infecção, quando RABV já está sendo eliminado pela saliva, os neurônios entram em processo de exaustão, acompanhado de disfunções e danos estruturais neuronais (LI, SARMENTO e FU, 2005; SCOTT et al., 2008; JACKSON et al., 2010). Estudos anatomopatológicos demonstram que animais e humanos infectados com RABV, comparativamente a outras encefalites virais, apresentam inflamação e destruição neuronal minimas (MORIMOTO et al; 1999; WANG et al., 2005; JACKSON et al., 2008), sendo estes indícios de evasão viral (LAFON, 2011). No entanto, há diferenças nestes achados, na dependência da amostra de RABV, sendo que amostras atenuadas induzem inflamação e morte neuronal (HOOPER et al., 2011; LAFON, 2011).

As infecções do SNC são controladas, em sua grande maioria, pela infiltração de linfócitos T, que são atraídos, juntamente com linfócitos B e macrófagos, ao local da infecção pelas citocinas e quimiocinas produzidas, a priori, pelas células residentes do SNC em resposta a infecção viral (ZHANG et al., 2008). Nos casos das infecções por RABV as células T são incapazes de debelar a infecção, pois são alvo de mecanismos de inativação induzidos pelo próprio vírus. Dentre estes podemos citar uma maior síntese de calcitonina, somatostatina, peptídeos vasointestinais e também pelo aumento de expressão de proteínas nos neurônios, como B7 Homolog (B7-H1) e FasL (Fas Ligand ou CD95L), que ao reconhecerem seus receptores nos linfócitos T, induzem a apoptose destes (BALOUL e LAFON, 2003;

BALOUL, CAMELO e LAFON, 2004; LAFON, 2005; LAFON et al., 2008; WEIHE et al., 2008). Interessante a ser notado é que o aumento de expressão de B7-H1 ocorre pela presença de moléculas como fator de necrose tumoral alfa (TNFα) e principalmente, pela presença de interferon beta (IFNβ), uma citocina a ser liberada de forma precoce pelas células infectadas por ter eficiente ação antiviral, ou seja, RABV dribla este mecanismo imune em favor da sua replicação (SARKAR e SEN, 2004; LAFON, 2011).

Após a penetração de RABV nos neurônios, os Toll-Like Receptors (TLRS) e RIG-I-Like Receptors (RLRs) são responsáveis por sinalizarem a presença viral e por iniciarem os mecanismos de defesa imediatos e subsequentemente, a resposta imune celular especifica (PREHAUD et al., 2005; LI et al., 2011). Este mecanismo de sinalização é essencialmente realizado pela liberação de citocinas e quimiocinas (MURPHY, 1977; ZLOTNIK e YOSHIE, 2000; MELCHJORSEN, 2003).

Citocinas e quimiocinas são proteínas produzidas e liberadas, predominantemente, pelas células mononucleares fagocíticas e por outras células apresentadoras de antígenos, além dos linfócitos T. O tipo de citocina produzida irá determinar o perfil da resposta gerada, podendo ser citotóxica, humoral, mediada por células ou alérgica (BORISH e STEINKE, 2003). As citocinas agem de maneira integrada, uma vez que a liberação de uma estimula a produção e secreção de outras moléculas, resultando no recrutamento de células inflamatórias para o SNC, a indução de uma resposta imune especifica, e consequentemente, influenciando na neuroinvasividade viral (FABER et al., 2004).

A infecção pelo RABV leva principalmente a produção de altos níveis de Interferon tipo I, especialmente de IFN-beta (IFNβ), após reconhecimento e ativação dos TLRs e RLRs (NAKAMICHI et al., 2004; FAUL et al., 2010) sendo esta citocina importante entre a ligação da resposta imune inata com a adquirida (BIRON, 2001). A infecção leva a ativação dos fatores reguladores de IFN (IRF), IRF-3 e IRF-7 que por sua vez passam a estimular genes sensíveis ao IFN (ISGs)

(TANIGUCHI e TAKAOKA, 2002), como OAS1 que também exerce ação antiviral (CHOPY et al., 2011). Embora a produção de IFNβ seja um mecanismo de defesa importante para a célula infectada, pois exerce efeito antiviral e induz uma cascata de ativação, as amostras altamente patogênicas conseguem utilizar a expressão de B7-H1, que é uma molécula IFN-dependente, para impedir o reconhecimento dos neurônios infectados por parte dos linfócitos T (LAFON et al., 2008).

Contrariamente `as amostras altamente patogênicas, as amostras atenuadas de RABV levam a uma elevada expressão de citocinas (WANG et al., 2005; SUGIURA et al., 2011; ZHAO et al., 2011), maior infiltração de células inflamatórias no SNC, bem como aumento na permeabilidade da barreira hemato-encefalica (BHE) relacionado à presença de IFN-gama (IFNγ), estando estes fatores associados ao *clearance* viral (PHARES et al., 2007; ROY et al., 2007; ROY e HOOPER, 2007; SPINDLER e HSU, 2012) e consequentemente, a uma maior chance de sobrevivência (PHARES et al., 2006; KUANG et al., 2009).

A duração da expressão dos genes relacionados à resposta imune varia de acordo com a amostra viral e o acúmulo de células inflamatórias no SNC, podendo ser deletério, uma vez que, a persistente migração de células T e neutrófilos para o interior do SNC acarretam em liberação de mais radicais livres e citocinas pró-inflamatórias que causam destruição do tecido nervoso (FU et al., 1993; NIU et al., 2011). Em estudo realizado por Zhao e colaboradores (2009), uma amostra atenuada de RABV foi geneticamente modificada para expressar três quimiocinas, CCL3, CCL5 e CXCL10. Demonstrou-se que os animais inoculados com o vírus expressando CCL3 apresentaram menor severidade do quadro clinico quando comparado ao vírus não modificado, no entanto, nos animais inoculados com CCL5, e principalmente com CXCL10, o quadro clínico foi de grave doença neurológica, explicado pela expressão continua destas quimiocinas, que ocasionaram uma penetração e acúmulo massivo de células inflamatórias decorrente do aumento da permeabilidade da BHE, confirmando que a expressão de quimiocinas é importante para o

controle da infecção, mas pode ser nocivo quando excessiva e não controlada.

Abordagem terapêutica na raiva

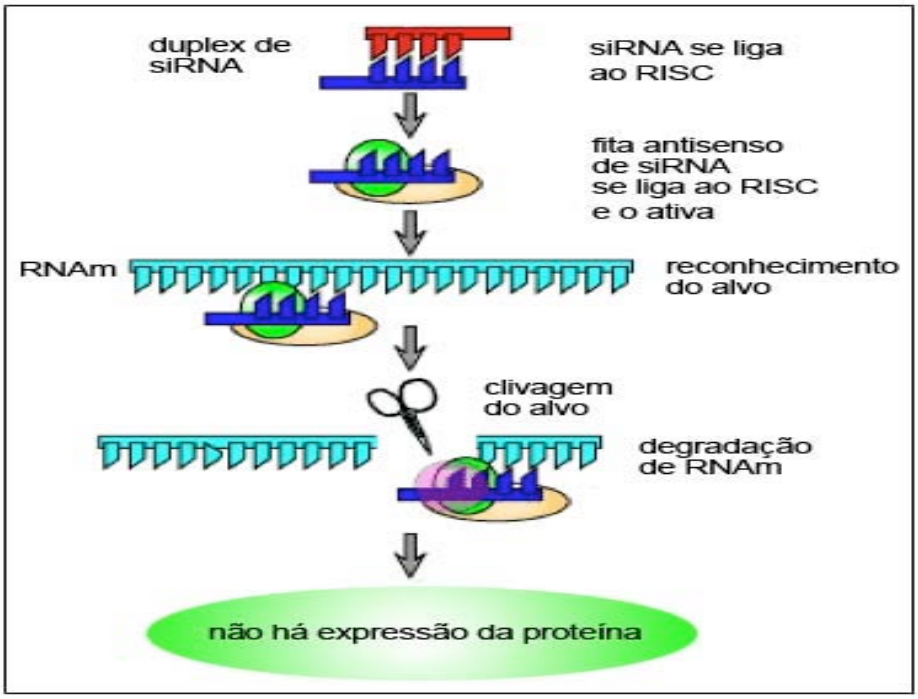
As primeiras tentativas terapêuticas na raiva ocorreram em 1889, com a utilização de soro hiper imune, sendo que várias surgiram posteriormente com uso de interferons, indutores de interferons e substâncias imunomoduladoras, no entanto, sem qualquer sucesso (HARMON e JANIS, 1975; BAER et al., 1977; MORENO et al.,1979; SODJA e HOLY, 1980; PEPIN e BLANCOU, 1985; BUSSEREAU,1986a; BUSSEREAU, 1986b ; SODJA, 1986; CHAVALI e CAMPBELL, 1987), sendo os únicos relatos de sobreviventes, aqueles submetidos a terapia pós-expositiva baseada no uso associado de vacinas e soro hiperimune (JACKSON, 2014).

No entanto, no ano de 2004, uma jovem americana, após dar entrada em um hospital em Wisconsin com alterações neurológicas, um histórico de agressão por um morcego, e presença de anticorpos anti-rábicos soroneutralizantes sobreviveu sem ter recebido qualquer tratamento pós-expositivo baseado em vacina e sorohiperimune (JACKSON, 2009). O protocolo terapêutico aplicado neste caso ficou conhecido como "Protocolo de Milwaukee" sendo sua primeira versão baseada em indução do estado de coma pela administração de benzodiazepínicos e barbitúricos, bem como na aplicação de ketamina intravenosa, ribavirina e amantadina (WILLOUGHBY et al., 2005). Este protocolo, a principio, causou grande alvoroço e expectativas positivas na comunidade médica, no entanto, após 10 anos, ele não apresentou resultados satisfatórios e seu principal componente, o coma induzido, foi abandonado por comprovada falta de eficácia e potenciais efeitos adversos (HEMACHUDA et al, 2006; JACKSON, 2013). Estudos *post-mortem* em amostras de pacientes submetidos ao protocolo, demonstraram uma grande quantidade de vírus da raiva no tecido cerebral, atestando a ineficácia deste em prevenir a morte ou mesmo em reduzir o nível de infecção no cérebro (McDERMID et l., 2008; HUNTER et al., 2010; MAIER et al., 2010).

Foram relatados pelo menos 26 casos em que o protocolo de Milwaukee foi utilizado sem sucesso (HEMACHUDHA et al., 2006; JACKSON, 2009; JACKSON, 2010; JACKSON, 2013), ressaltando a necessidade de terapias novas e comprovadamente eficientes.

Nova abordagem terapêutica

O RNA de interferência (RNAi) é um mecanismo endógeno de inibição ou silenciamento pós-transcricional, descoberto e descrito por Napoli et al. (1990) em trabalhos com flores e elucidado por Fire et al. (1998). O mecanismo se baseia no princípio de que uma fita dupla de RNA, composta por cerca de 20 pares de bases (siRNA), e com sequência anti-senso complementar a um RNA mensageiro (RNAm) alvo, seja capaz de desencadear a clivagem deste, impossibilitando assim a sua decodificação. Internalizado pela célula, já no citoplasma, o siRNA é capturado por um complexo ubiquitário de proteínas denominado RISC (complexo silenciador induzido por RNA), o qual possui um domínio de ligação RNA dupla-fita e um domínio com atividade de ribonuclease. A ligação siRNA-RISC ativa o complexo e leva à separação da fita-dupla de RNA e à degradação do RNAm alvo que está ligado com a fita anti-senso do siRNA, impossibilitando sua transcrição e consequentemente a síntese protéica (AKHTAR e BENTER, 2007) (Figura 1).



Fonte: Durymanova-Ono, 2010

FIGURA 1- Esquema gráfico do mecanismo de ação do RNAi.

O mecanismo de RNAi tem um papel importante na defesa celular contra a agressão viral, além de outras funções importantes, como a mobilidade de elementos genéticos e a regulação da expressão de genes relacionados ao desenvolvimento nos animais (MESHRAM et al., 2013). Nas últimas décadas, o potencial geral deste mecanismo tem estimulado estudos quanto a utilização de siRNA e microRNA no tratamento de doenças não infecciosas, como problemas cardíacos, doença de Huntington, diversos tipos de câncer e tumores, problemas capilares, doenças oculare e artrite autoimune (ARAÚJO et al., 2010; ZHENG et al., 2010; CHEN e ZHAORI, 2011), bem como em doenças infecciosas como dengue (SANCHEZ-VARGAS et al., 2004; MUKHERJEE e HANLEY, 2010), problemas respiratórios causado por Vírus Sincicial Respiratório, (CHANG et al., 2007), Influenza (SCULL e RICE, 2010), tuberculose (JAYASWAL et al., 2010), SARS (WANG et al., 2010), AIDS (CHEN e ZHAORI, 2011) e Herpes Simplex tipo 2 (KATAKOWSKY e PALLISER, 2010) dentre outras.

Na raiva foi testado *in vitro* e *in vivo,* sendo as sequências- alvo mais comuns os genes da nucleoproteína (N), da glicoproteína (G) e/ou

da polimerase (L) (ISRASENA et al., 2011). Um dos principais desafios quanto ao uso do siRNA, consiste no desenvolvimento de um sistema de transporte desta molécula para o interior da célula-alvo, uma vez que a polaridade e tamanho não permitem que ela passe pela membrana celular com facilidade. Dois tipos de transporte são descritos, os vetores virais, como os adenovírus, ou plasmídeos de DNA capazes de carrear o shRNA (*short-harpin RNA*), que no interior da célula hospedeira se transformará em siRNAs, e os vetores não virais como polímeros, lipossomos catiônicos e complexos peptídicos (CHEN e ZHAORI, 2011; GUPTA et al., 2012; YANG et al., 2012).

Em todos os estudos publicados, siRNA demonstrou em alguma instância, inibir a replicação viral (BRANDÃO et al., 2007; ISRASENA et al., 2009; GUPTA et al., 2012; YANG et al., 2012; MESHRAM, 2013), o que ressalta o potencial de tal tecnologia como uma alternativa terapêutica na raiva. No entanto, algumas dificuldades ainda devem ser transpostas, como um método mais eficiente de carreamento e principalmente, a limitação do silenciamento frente a amostras de rua do virus, uma vez que estas possuem alta taxa de variabilidade genética (ISRASENA et al., 2011).

CAPÍTULO 2

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Immune response evaluation in mice infected with wild-type strains of rabies virus

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Abstract

Rabies is a lethal infectious disease that causes 55 thousand human deaths per year, with most of them occurring in African and Asian countries. Dogs, bats or other carnivores can transmit the virus, especially through bites and scratches. The host immune response is essential to avoiding viral progression and promoting viral clearance. Cytokines and chemokines are crucial in the development of an immediate antiviral activity and for the attraction of NK cells, monocytes, B and T lymphocytes; in order to succeed the infection rabies virus has to evade this immune response. The virus' capacity for evasion is correlated with rabies virus pathogenicity, with the most highly pathogenic strains being the most efficient in hijacking the host's defense mechanisms. The purpose of this study was to evaluate the gene expression of a set of cytokine and chemokine genes related to the immune response in the brains of mice inoculated i.m or i.c with two wild strains of rabies virus, one from dog (V2) and another from vampire bat (V3). The results demonstrated that the gene expression profile is intrinsic to the

specific RABV variant. More efficient virus evasion, related to IFNβ inhibition, was observed in the group infected with a high lethal dose. The precocious production of cytokines and chemokines seems to be more important than their levels of expression for rabies survival.

Key words: rabies, immune response, gene expression, dog virus, bat virus, wild strain

1. Introduction

Rabies is a zoonotic, highly lethal and neglected disease that has been affecting humanity for more than 4.000 years (Knobel et al., 2005). It is estimated that around 55.000 persons die from rabies each year, killing more than dengue, yellow fever and Japanese encephalitis. Even so, it ranks low on the World Health Organization's priority list (Hemachudha et al., 2002). Data obtained from global mortality reports estimate that one individual dies from rabies each 10 minutes and another 300 are exposed to the virus (Fooks et al., 2009). Domestic dogs are mainly responsible for rabies transmission, specially in Asian and African countries but other wild mammals such as bats, non-human primates, foxes and wild dogs can be responsible for human cases and some studies have shown that those species are reservoirs for unique variants of rabies virus (RABV) (Ito et al., 2001; Schneidder et al., 2009; Brasil, 2010; Aguiar, 2011).

RABV, a *Lyssavirus* belonging to the *Rhabdoviridae* family, causes acute encephalitis and has the highest case-fatality rate among infectious diseases, being almost invariably lethal in 100% of cases (Fooks et al., 2014). It is transmitted in most cases by the saliva of an infected animal through bites, scratches or contact with mucous membranes. It invades the central nervous system (CNS), binding to receptors present at neuromuscular junctions or in the sensory nerves (Wang et al., 2005). It reaches the spinal cord via retrograde axonal transport and then the brain, where intense replication

occurs followed by centrifugal dissemination, allowing the virus to be released through salivary glands.

Viral replication occurs in the cell bodies of neurons. The viral proteins can be detected in dendrites and viral spread occurs through transneuronal transference and relies upon the structural integrity of the neurons. Studies conducted in rat motor neuron cultures show that cells infected with RABV never die, and *in vivo* studies demonstrate that motor neurons are preserved for up to four days after infection (Ugolini, 2010; Klingen et al., 2008; Guigoni and Coulon, 2002). In the later phase of infection, when RABV is already being eliminated in the saliva, host cells die due to dysfunction and neuronal structural damage (Jackson et al. 2010, Scott et al. 2008). Pathological studies have shown that animal and human brains infected with RABV have encephalitis, but with minimal neuronal loss and varying degrees of inflammation (Wang et al., 2005; Jackson et al., 2008; Hemachudha et al., 2013), demonstrating that the virus has mechanisms to evade the host immune response (Lafon, 2011).

The uninfected CNS has intact endothelial tight junctions, low expression of adhesion molecules, which promote interactions with blood stream leukocytes, and microglia maintained in a non-activated state by direct contact of the CD200 receptor (CD200R) with its ligand CD200, which is expressed in healthy neurons. The production of neurotrophins and anti-inflammatory cytokines by astrocytes and meningeal cells helps to maintain the lack of immunological activity in the CNS (Griffin, 2003; Ransohoff and Cardona, 2010). However, as soon as a pathogen gains access to the CNS this dynamic is changed, and an immediate immune response takes place. After RABV is recognized by the Toll Like Receptors (TLRs) and RIG- I- Like Receptors (RLRs) that are present in neurons (Prehaud et al., 2005), interferon beta (IFNβ), the first cytokine to be produced at high levels, is a major antiviral protein and is also responsible for

initiating a cascade of transcription of several genes such as interferon regulatory factor 3 (IRF3) and IRF7 which activate interferon stimulated genes (ISG) (Taniguchi and Takaoka, 2002; Masatani et al., 2010) such as OAS1, another antiviral protein that works in a synergic way with IFNβ, each exerting its activity by inhibiting viral transcription, translation, protein synthesis and viral assembly (Chopy et al., 2011). The importance of these immediate cytokines has already been characterized in RABV infection (Nakamichi et al., 2004; Faul et al., 2010). IFNβ also links the innate and acquired immune response (Biron, 2001; Faber et al., 2004; Li et al., 2011). Besides IFNβ, stressed or damaged neurons also synthetize interferon gamma (IFNγ), interleukin 6 (IL6), chemokine (C-C motif) ligand 21 (CCL21) and chemokine (C-X3-C motif) ligand 1 (CX3CL1), the latter of which has receptors on macrophages and glial cells that are soon activated and produce several other cytokines and chemokines such as IL1, IL6, IL12, TNFα, CCL4, CCL5, CCL7 and chemokine (C-X-C motif) ligand 10 (CXCL10). These factors lead to upregulated expression of MHC by microglia and also increase the expression of adhesion molecules that promote interactions with circulating leukocytes and also promote the opening of the blood-brain-barrier, especially CXCL10 (Wang and Shuaib, 2002; Griffin, 2003). A study conducted by Mansfield et al. (2008) in mice infected with European Bat Lyssavirus type 2 demonstrated that animals with high expression of CXCL10, IL6 and IFNγ had a massive invasion of T cells in the CNS with a consequent robust immune response. The duration of gene expression related to the immune response varies according to the viral sample and may even be harmful beyond a certain point because it increases the release of reactive oxygen species that promote tissue destruction (Fu et al., 1993; Niu et al. 2011). A study by Zhao et al. (2009) with attenuated RABV genetically modified to express CCL3, CCL5 and CXCL10, important chemokines in monocyte, neutrophil and lymphocyte activity,

demonstrated that continuous expression of CCL5 and CXCL10 caused worse neurological signs when compared to CCL3, a group that animals had a mild clinical presentation.

The infiltration of inflammatory cells into the CNS includes all components of the cellular immune response such as NK, antigen specific CD 4^+ and $CD8^+$ T cells, B cells and macrophage (Griffin, 2003). This infiltration also occurs during RABV infection, but after a few days it disappears in case of highly pathogenic strains (Lafon, 2011). The most important cytokines produced during this phase are IFNγ, IL4 and IL10, and also specific Ab are produced by B cells (Griffin, 2003). CNS infections are controlled, in most cases, by infiltrating T cells, such as in of West Nile infection, especially by $CD8^+$ activity (Zhang et al., 2008). However, in RABV infections, specifically due to highly pathogenic strains, although T cells are activated normally in the periphery (Roy and Hooper, 2007) they do not determine the outcome as shown in a study conduced in mice lacking T cells (Lafon, 2005). This inefficacy occurs because the virus can induce a great production of calcitonin, somatostatin and vasointestinal peptides in infected cells, limiting the activity of the T cells (Weihe et al., 2008), or can even induce T cell destruction by the increased expression of molecules such as B7 Homolog (B7-H1) and Fas Ligand (FasL or CD95L) in the neurons, which, in contact with their receptors, Fas (CD95) and Programmed Cell Death Protein-1 (PD-1), present on activated T cells, induce apoptosis (Lafon et al. 2008). This demonstrates that a natural mechanism used by the host to control the inflammatory response in the brain can also act in the virus' favor to delay its replication and subvert the immune response (Griffin, 2003; Sarkar and Sen, 2004; Lafon, 2011). Immunohistochemical studies of RABV-infected brains and spinal cord demonstrated that neurons showed a large amount of antigen with intact morphology while migrating T cells showed apoptosis process (Baloul and Lafon, 2003;

Lafon, 2008; Fernandes et al., 2011). In contrast to the highly pathogenic strains, T cells are important in controlling infections caused by attenuated viruses, in which infected neurons are successfully eliminated by apoptosis and T cells remain intact (Hooper et al., 2011; Lafon, 2011; Sugiura et al., 2011), indicating that T cells effective activity is intrinsically associated with the pathogenicity of the RABV. The sequence of the RABV glycoprotein (G) has also been shown to be a determinant in neuron apoptosis (Faber et al., 2002; Prehaud et al., 2010). In particular, the last four amino acids of the called cyto-G portion of the protein, which targets the PDZ domain in the host cell and leads to destabilization of the complex formed by PDZ and its ligands, may cause profound changes in important cellular signaling pathways that can lead to apoptosis (Aarts et al., 2002; Hou et al., 2010)

Differences in the pathogenicity of RABV strains are not only associated with viral replication or the cellular infection rate, but with the level and duration of cytokine expression in addition to the induction of immune cells apoptosis, which therefore guarantees the integrity of the neurons (Zhao et al., 2009). Several studies have shown that attenuated RABV samples lead to high expression of cytokines, an increase in BBB permeability related to the presence of IFNγ, and infiltration of inflammatory cells into the CNS; together all these factors enable the viral clearance (Phares et al., 2006; Phares et al., 2007; Roy et al., 2007; Roy and Hooper, 2007; Spindler and Hsu, 2012), enhancing the chances of survival (Kuang et al., 2009). Human survivors of rabies had in common the fact that neutralizing antibodies (VNA) were detectable in their cerebrospinal fluid (CSF) at an early stage in the disease, something that does not happen in unfavorable outcomes when VNA are only detectable in the very end stage (Jackson, 2014). Viral clearance is essential for recovery in any neurological infection and neutralizing antibodies play an important role in this process, but early penetration

of VNA as well as B cells into the CNS depends on early opening of the BBB (Roy et al., 2007) and, as already considered, is related to the pathogenicity of the RABV strain. This premature enhancement in BBB permeability is not often observed what can be concluded from the amount of deaths and survivals in rabies.

Considering the crucial role of cytokines and chemokines in immune response regulation (Zlotnik and Yoshie, 2000; Borish and Steinke, 2003) the aim of this present study was to evaluate the expression of selected cytokines and chemokines in CNS of mice experimentally infected with two different wild-type strains, one from dog (V2) and one from a vampire bat (V3).

2. Materials and Methods

2.1 Animals and Virus

Female C57/BL6 mice, 4 to 6 weeks old, were provided by Cemib (UNICAMP animal facility) and used for experimental rabies inoculation. The animals were kept in special containers and received sterile water and irradiated food "*ad libitum*".

Dog rabies virus and hematophagous bat rabies virus, both wild strains, were isolated from human cases and characterized as variant 2 and variant 3, respectively.

2.2 Experimental design

Experiment 1: mice were separated in two main groups and inoculated intramuscularly (i.m) in the right hind limb with 100 microliters (µl) of 80 LD_{50} (80V2) of variant 2 inoculum or 40 LD_{50} (40V3) of variant 3 inoculum. For each LD_{50}, the animals were separated in 3 groups with 8 animals each; one group was maintained for clinical evaluation during a 30 day period and the two others were euthanized at 5 and 10 days post-inoculation (d.p.i) for brain collection.

Experiment 2: mice were inoculated intramuscularly (i.m), in the right hind limb with 100µl of 40 LD_{50} (40V2) of variant 2 inoculum. The animals were then separated in 3 groups with 6 animals each; one group was maintained for clinical evaluation during a 30 day period and the two others were euthanized at 5 and 10 d.p.i for brain collection.

Experiment 3: In order to evaluate the immune response in the final stage of the disease, two groups of 8 animals were inoculated via intracerebral route (i.c) with 30 µl of variant 2 (V2) or variant 3 (V3) inoculum with the same viral titer ($10^{-6.66}$ DL_{50}/0.03 mL). The animals were observed during a maximum period of 30 days and brains were collected immediately after death.

For each group a control was established in which animals were inoculated only with the viral diluent by the same route as the infected ones, whether i.m or i.c.

Animals in all the groups were weighted and evaluated daily for the onset of clinical signs such as ruffled fur, hunching back, hypo/hyper excitability, paralysis of one or both hind limb or tetraplegia (Chopy et al., 2011).

The animal study was approved by the São Paulo State University Ethics Committee (registration number 238/2008), which follows the guidelines established by COBEA-The Brazilian Society of Laboratory Animal Science.

2.3 RNA extraction and RT- qPCR

Brain tissues RNAs were extracted with the Invitek® kit and stored at -80ºC. The reaction for cDNA synthesis consisted of 1 µg of extracted RNA, 1 µl of Oligo-DT primer (Invitrogen®) and 1 µl of SuperScript II (Invitrogen®) according to manufacturer's instructions. The RT-qPCR reaction was performed with 2 µl of 1/50 diluted cDNA, 1 µl of 0.1 µg of each primers and Master Mix Syber Green (Promega®) in a final volume of 25 µl according manufacturer's instruction. Primers for the 18S

murine genes were supplied by IDT® and used as keep-housing gene and primers for the RABV N protein gene were manufactured as described previously (Soares et al., 2002). Mouse Quantitect ® Primer Assay from Qiagen® were used to evaluate the expression of *CCL2, OAS1, IL2, IL6, IL12, TNFα, IFN γ, IFNβ, CXCL10, CD200R e IGF1 genes.*

All thermal cycling and detection was performed using an Applied Biosystems StepOne Fast® (ABI7500 Fast) thermal cycler employing a thermal profile of 40 cycles of 50°C for 20 s, 95°C for 10 min., 95°C for 15 s and 60°C for 1min.

2.4 Data Analyze

Graph-Prism® 5.0 and Instat® software were used as analysis tools. The Kruskal-Wallis test with $p<0.05$ was selected for global evaluations of cytokine and chemokine gene expression, and Two-tail Mann-Whitney test to compare the expression of a single cytokine/chemokine at 5 and 10 days post-inoculation. Values of p between 0.05 and 0.1 were considered as significance tendence.

3. Results and discussion

3.1 Intramuscular inoculation (Experiments 1 and 2)

The incubation period was 9 days, shown to be dose and variant-independent. A study published by Charlton et al. (1987) demonstrated that skunks inoculated intramuscularly with several dilutions of street rabies virus only presented differences in the incubation period after the second 10-fold dilution, as in the present study, in which the same incubation period was observed even for the group inoculated with 40 LD_{50} of V2. The evolution period was 8, 8 and 13 days and mortality rate was 100%, 60% and 66%, respectively, for 80V2, 40V3 and 40V2 with no statistical differences for the evolution

period. This demonstrates that after the entry of virus particles in the CNS, there is no difference between dog and bat variants regarding the time required for disease progression. A recent study about the major characteristics of human rabies cases due to bat and dog variants showed that after the onset of clinical signs there was no difference in the survival time between the two variants (Udow et al., 2013). Our survival analysis showed a difference between 40V3 versus 40V2 ($p=0.003$) and 40V2 versus 80V2 ($p=0.01$) (Fig. 1).

When *N gene* expression was assessed no difference was found between 5 and 10 d.p.i, among the groups. As expected and according to the progression of the infection, *N gene* expression was higher at 10 days compared to 5 days, but the only group that presented a significant difference between 5 and 10 days was the one inoculated with 80V2 ($p<0.05$) (Fig. 2). In contrast, in a study published by Chopy et al. (2011)**,** in mice inoculated by the intramuscular route with a lethal dose of CVS and sacrificed in different stages of clinical disease, when *N gene* transcripts of RABV were evaluated in those animals, no difference was observed in the total amount of virus between the different phases of the disease. This discrepancy can be justified by differences in the rabies virus strains used in each study, a laboratory adapted strain and a wild type strain. These data suggest that differences in *N gene* levels could be associated with the mortality rate observed in 80V2 in the present study, since a statistical difference in mortality was observed associated with higher *N gene* expression at 5 d.p.i compared to 10 d.p.i.

At 80V2 the expression of IFNβ at 5 d.p.i was higher compared to all other immune markers analyzed ($p< 0.001$). Its expression was decreased at 10 d.p.i, at which point IL12 ($p< 0.01$), among others, had high expression. Comparing each cytokine and chemokine individually at 5 versus 10 d.p.i, CD200R ($p= 0.001$), IL12 ($p=0.01$) and

IFNβ (p= 0.06) all had differences in their expression (Fig. 3A). As described before, IFNβ is one of the first cytokines to appear during rabies infection, as expected in infection process (Griffin, 2003; Steel et al., 2014), and counteracting such a response is one of the main mechanisms of viral evasion. The high expression of IFNβ at 5 d.p.i followed by a decrease at 10 d.p.i shows that the RABV strain was able to efficiently disturb the innate immune response, as described in other studies with highly virulent rabies viruses (Chopy et al., 2011). This efficient suppression of this early phase cytokine can be associated with the mortality result observed in this group.

In the 40V2 group, high expression of IL12 ($p<0.05$) at 5 d.p.i was observed, with a further discrete enhancement at 10 d.p.i ($p=0.09$) associated with higher expression of OAS1 ($p=0.01$) and IFNβ ($p=0.01$), although all the immune markers seemed to have an increased expression at this point compared to 5 d.p.i (Fig. 3B).

Animals inoculated with 40V3 had high expression of IFNβ ($p< 0.01$) and CCL2 ($p< 0.001$) at 5 d.p.i. and at 10 d.p.i no difference was observed for any cytokine or chemokine between groups and moments; however, when their expression was compared individually at 5 versus 10 d.p.i, TNFα ($p=0.0005$), IFNβ ($p=0.0002$), IL12 ($p=0.0047$) and CD200R ($p=0.0047$) were statistically increased at 10 d.p.i (Fig. 3C).

Comparing the results just for those immune markers with a statistically enhanced expression at 5 and 10 d.p.i in the 40V2 versus 40V3 groups, even though there was a similar mortality rate in both groups, at 5 d.p.i V2 had greater expression of IL12 ($p= 0.007$) while V3 had a more prominent expression of IFNβ ($p= 0.007$). At 10 d.p.i no difference was found. Considering that the animals were inoculated with the same lethal dose of both variants and had similar mortality rates, biologically the profile of cytokines and chemokines seems to show that V2 induces the expression of a larger number and intensity of those analyzed genes, especially at 10 d.p.i, when compared to

V3. A study performed in mice inoculated with same lethal dose, via i.c or i.m route, using a highly pathogenic Silver-Haired Bat rabies virus (SHBRV) or an attenuated CVS strain (B2C) showed that the number of genes up-regulated by the B2C strain was higher in both number and intensity. It is also interesting to note that the OAS1 gene was upregulated 4-fold when compared to IFNβ in the same group, 2-fold higher when compared to OAS1 expression and more than 6-fold higher when compared to IFNβ expression in the SHBRV group (Wang et al., 2005). Although both rabies virus strains used in the present study were classified as highly pathogenic, since they were isolated from human rabies patients, the gene expression of V2 and V3, even with same viral titer and same mortality rate, was biologically distinct, especially at 10 d.p.i related to the induced and upregulated gene expression of V2 compared to V3. These data in the present study show that the mortality rate does not appear to be associated with gene expression.

Comparing the same variant (V2) with different lethal doses, 80LD versus 40LD, and different mortality rates between those 2 groups ($p=0.01$), at 5 d.p.i 40LD had a higher expression of IL12 ($p< 0.0001$) and 80LD had stronger expression of IFNβ ($p= 0.01$); at 10 d.p.i IFNβ expression was higher in the 40LD group. A study by Chopy et al. (2011) characterizing the ability of the CVS RABV strain to trigger and thwart the type I IFN response in SKNSH cells showed that a higher multiplicity of infection (MOI) resulted in earlier detection of IFNβ, but the amount of input virus did not change the magnitude of the response, explaining, at least partially, why in the present study 80V2 had an earlier expression of IFNβ compared to 40V2. The mean *N gene* expression at 5 d.p.i in the 80LD group was more than 2-fold lower than in the 40LD group, even though it was inoculated with 2-fold more virus. This difference disappeared at 10 d.p.i, suggested by the high level of virus replication in this group at 10 dpi. It can be concluded that the

early high expression of IFNβ in the 80LD group was strong enough to delay virus replication at 5 d.p.i, but once viral evasion mechanisms were activated this inhibition became ineffective (Masatani et al., 2013). This can be noticed at 10 d.p.i, when *N gene* expression was equally high in both groups but IFNβ expression was at a lower level in the 80LD group. The same was noted in the 40LD group, which showed low IFNβ expression at 5 d.p.i and high *N gene* expression at the same period; even after there was an enhancement in IFNβ expression at 10 d.p.i, the level of the *N gene* was not lower than in the 80LD group, confirming data obtained in cultured cells that IFN I response effectiveness vanishes when a large number of cells are already infected (Chopy et al., 2011). It is interesting to notice that although OAS1 is induced by the presence of IFNβ and also has an antiviral activity, its high expression in the 40LD group at 5 d.p.i was not sufficient to inhibit viral replication as much as IFNβ. The high expression of IL12 at 40LD suggests that a lower viral dose induces a broader and earlier immune response once this cytokine is released by macrophages, dendritic cells and NK cells activated during an infectious process, which also explains the increased number genes expressing at 10 d.p.i in this group. In contrast, the 80LD group basically presented a marked initial antiviral response with no sign of early activation of other components of the immune response, evidenced by low expression of all analyzed genes at 10 d.p.i. All these results suggest that the inate immune respose can be important in rabies survival (Griffin, 2003; Li et al., 2008; Zhao et al., 2010).

3.2 Intracerebral inoculation (Experiment 3)

The mortality rate of animals inoculated by i.c route was 100% for V2 and V3 but analysis of the survival curves showed difference in disease progression between the two variants ($p=0.01$). The median incubation period was 8 days for V2 and 9 days for

V3 ($p=0.0002$), while the median evolution period for V2 was 4 days and only 1 day for V3 ($p<0.0001$). A study in India analyzing data from human rabies patients showed that the incubation period is not correlated with the rapidity of the clinical course of the disease (Solanki et al. 2009). Evaluation of RABV *N gene* expression also showed differences between the two variants, with the highest expression being observed with V3 ($p=0.01$) (Fig. 4). The short evolution period can be positively associated with the high *N gene* expression in the V3 group.

The immune markers analyzed in both variants showed increased expression of the same cytokines and chemokines, with equal p values. These were IFNγ ($p<0.001$), CXCL10 ($p<0.001$), TNFα ($p<0.05$) and CCL2 ($p<0.001$) (Fig. 5). The major activity of chemokines (CXCL10 and CCL2) is to modulate the trafficking of T cells to the CNS, and they are also important in promoting viral clearance. Studies made with different virus agents such as herpes simplex and West Nile showed that the deletion of the gene responsible for CXCL10 expression led to a poor infiltration of T cells, as well as inefficient viral control associated with more severe disease. However, like a dual-edged sword, an excess of T cells can also be deleterious because it causes greater cytotoxicity and inflammation (Zhao et al. 2009; Niu et al. 2011). Several studies made *in vivo* or in *in vitro* models demonstrated that CXCL10 is present and upregulated after RABV infection (Wang et al., 2005; Mansfield et al., 2008; Zhao et al., 2013). This chemokine has been shown to play an important role in BBB permeability, which seems to be essential in the clearance of RABV from the CNS since it allows the entry of immune effector cells (Kuang et al., 2009). Although studies made by Wang (2005) and Kuang (2009) described enhanced expression of CXCL10 in models infected with attenuated RABV but in not in one infected with wild type RABV, the results of the present study revaled a statistically significant upregulation of CXCL10 and TNFα in

animals infected with wild type strains. In a study made in brains of human rabies patients correlating the presence of TNFα and IL1 (proinflammatory cytokines) with the presence of RABV in immunohistochemistry assay showed that the presence of these cytokines was positively correlated with brain inflammation, but no association was established between viral load and higher levels of TNFα (Solanki et al., 2009).

When these four increased cytokines and chemokines were compared between the V2 and V3 groups, the expression of CCL2 ($p= 0.01$) and CXCL10 ($p=0.01$) was higher in V2 than in V3. The statistically high expression of these chemokines, although correlated with T cell attraction and increase in BBB permeability, can possibly be related to the lower levels of the *N gene* product present in the V2 group but they are not associated with a decreased mortality rate, since it was 100% in both groups. In a study made by Mansfield et al. (2008), high expression of CXCL10, IFNγ and IL6 in mice in a clinical phase of encephalitis due to European Bat Lyssavirus type 2 was not related to survival. Altogether, it can be concluded that even with high expression of cytokines and chemokines involved in the inflammatory process as well as mediating the specific immune response, a substantial innate response was still insufficient to control infection and prevent death.

4. Conclusions

In the present work a more efficient virus evasion mechanism was observed in the group infected with a higher lethal dose (80V2). The evasion mechanism was characterized by IFNβ inhibition with consequent downregulation of analyzed cytokines/chemokines associated with high *N gene* expression.

Variants 2 and 3, inoculated with same lethal dose by the intramuscular route, presented the same mortality and clinical features but different gene expression profiles, indicating that the immune response is intrinsic to the RABV variant.

The precocious production of cytokines and chemokines seems to be more important than their levels of expression for rabies survival, as observed in moribund animals that, although expressing high levels of these analyzed cytokines and chemokines related to the host-specific immune response, succumbed to the disease.

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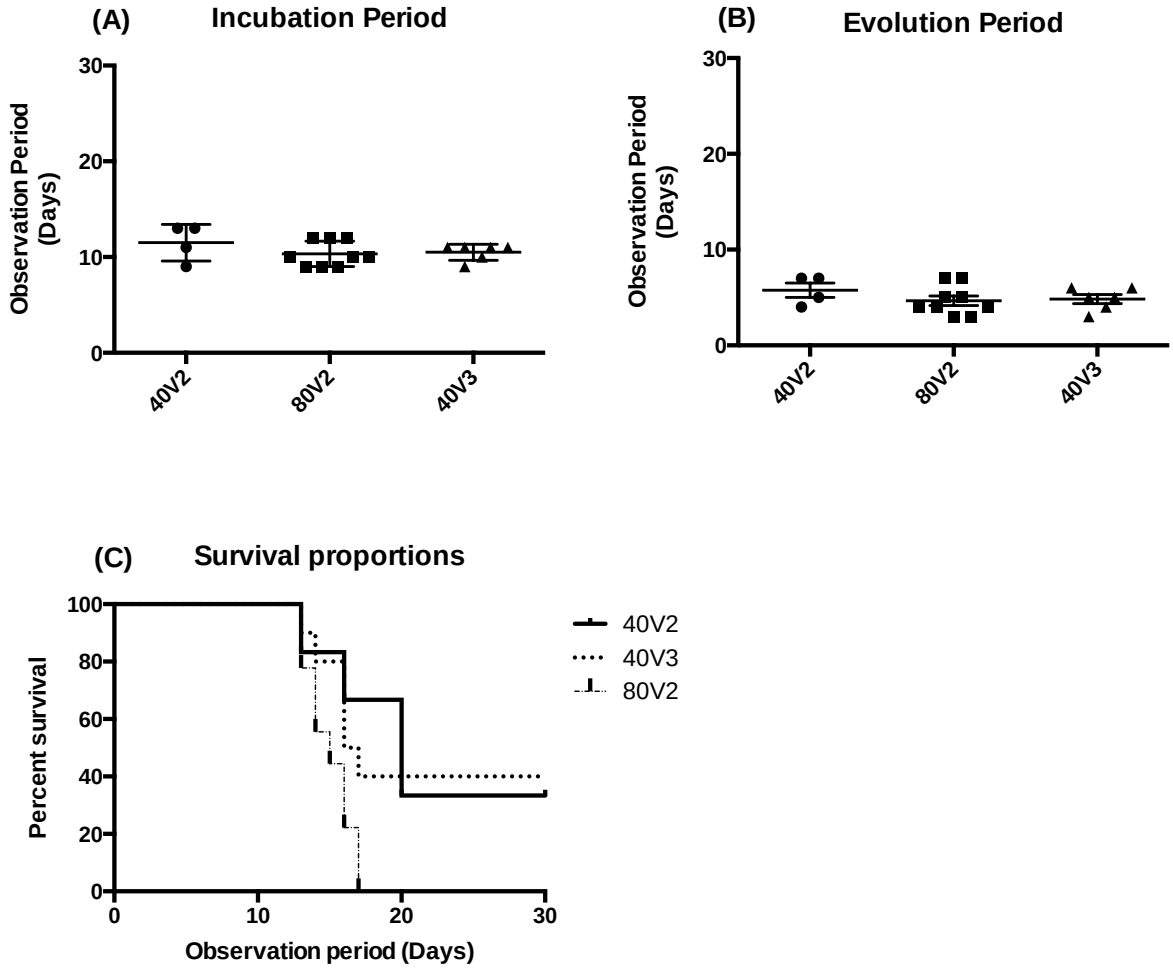

Figure 1. (A) Incubation period of 40V2, 40V3 and 80V2 groups (i.m inoculation); Kruskal-Wallis test was used to calculate differences between the groups. No statistical difference was found. (B) Evolution period of the three groups; Kruskal-Wallis test showed no statistical difference ($p > 0.05$) between the groups. (C) Survival analysis between groups with a 30 day observation period; Mantel-Cox test demonstrated a statistical significance between 40V3 versus 40V2 ($p=0.003$) and between 40V2 versus 80V2 ($p=0.01$).

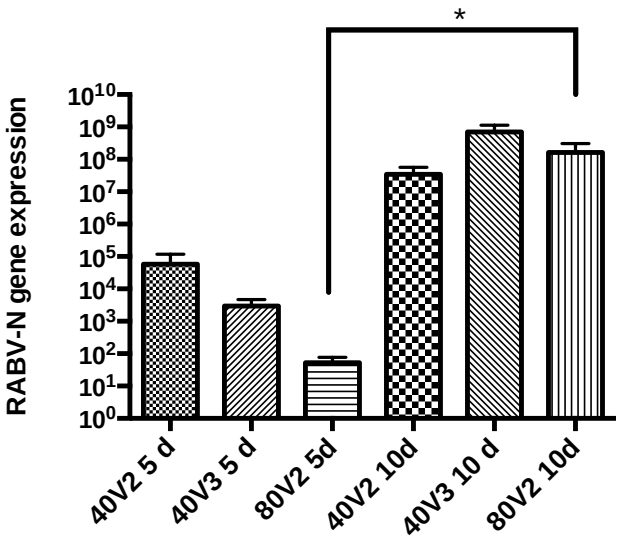

Figure 2. RABV *N gene* expression in the brains of mice infected with 40V2, 40V3 or 80V2. Kruskal-Wallis test was applied to compare the results between different groups at 5 and 10 d.p.i. There was no difference between the groups at the same period, 5 d.p.i or 10 d.p.i. However, 80V2 showed a significant difference (*$p < 0.05$) at 5 versus 10 d.p.i.

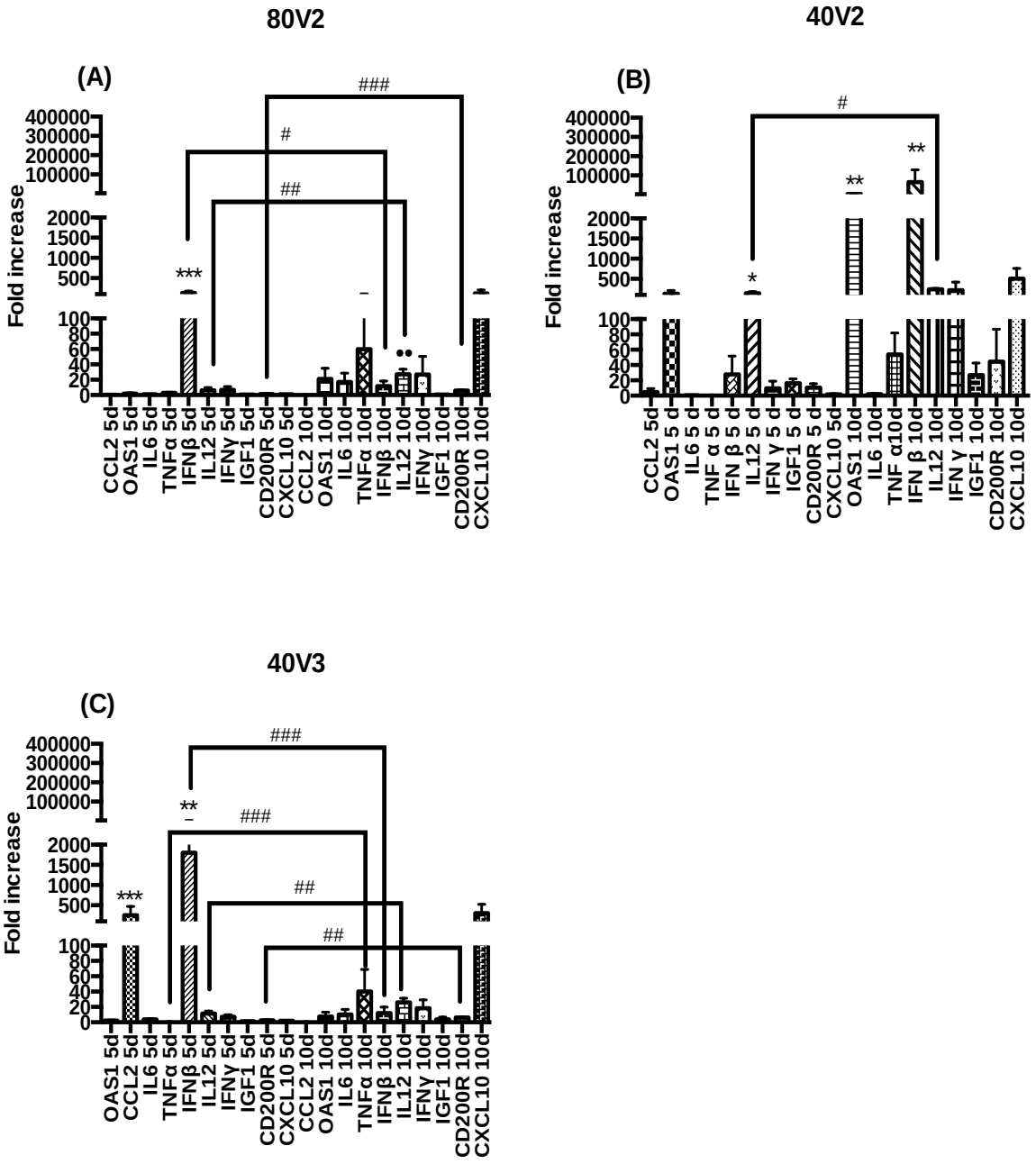

Figure 3. Relative gene expression of cytokines and chemokines in different groups (40V2, 40V3 and 80V2) at 5 and 10 d.p.i. Kruskal-Wallis test was applied to analyze the global results and Mann-Whitney two-tail test was applied to make individual comparisons at 5 versus 10 d.p.i. (A) Expression at 5 d.p.i and 10 d.p.i in the 80V2 group; IFNβ was highly expressed at 5 d.p.i (***$p < 0.001$), at 10 d.p.i IL12 had the higher expression (**$p<0.01$) and comparing 5 d.p.i versus 10 d.p.i CD200R (###$p=0.001$), IL12 (##$p=0.01$) and IFNβ (#$p=0.06$) showed differences in expression.

(B) Expression at 5 d.p.i and 10 d.p.i in the 40V2 group; IL12 (*p<0.05) had the higher expression at 5 d.p.i and OAS1 (**p=0.01), IFNβ (**p=0.01) at 10 d.p.i; comparing 5 d.p.i versus 10 d.p.i IL12 (#p=0.09) showed a slight increase. (C) Expression at 5 d.p.i and 10 d.p.i in the 40V3 group; IFNβ (**p<0.01) and CCL2 (***p<0.001) had higher expression at 5 d.p.i. No difference was observed at 10 d.p.i, although in the comparison between 5 d.p.i versus 10 d.p.i TNFα (###p=0.0005), IFNβ (###p=0.0002), IL12 (##p=0.0047) and CD200R (##p=0.0047) showed a higher expression at 10 d.p.i.

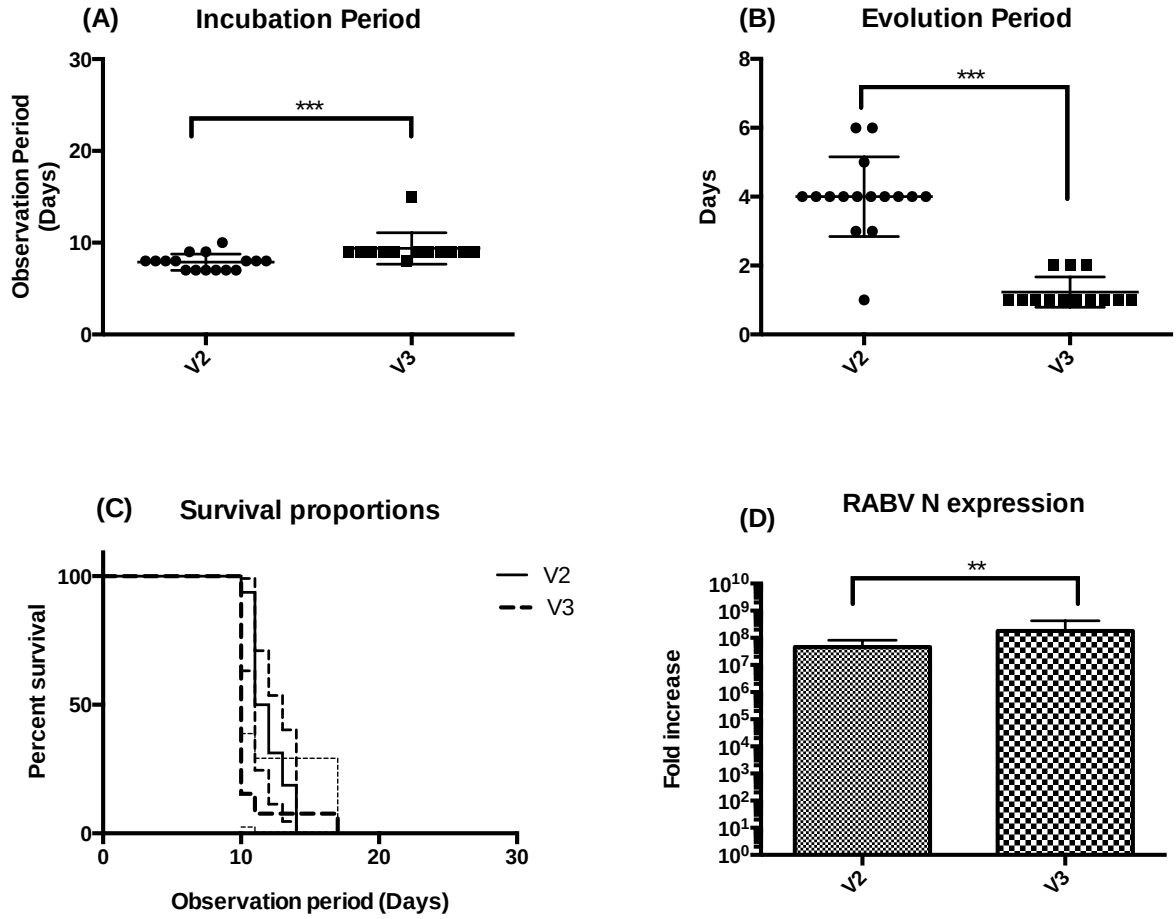

Figure 4. (A) Incubation period of V2 and V3 groups (i.c inoculation); Mann-Whitney two-tail test showed a significant difference between the groups (***p=0.0002). (B) Evolution period of V2 and V3 groups; Mann-Whitney two-tail test also showed a statistical difference (***p < 0.0001) between the groups. (C) Survival analysis curve between V2 and V3 within the 30 day observation period; Mantel-Cox test demonstrated a statistical significance between the two groups (**p<0.01). (D) Relative RABV *N gene* expression between V2 and V3; Mantel-Cox text showed a higher expression in the V3 group (** p=0.01).

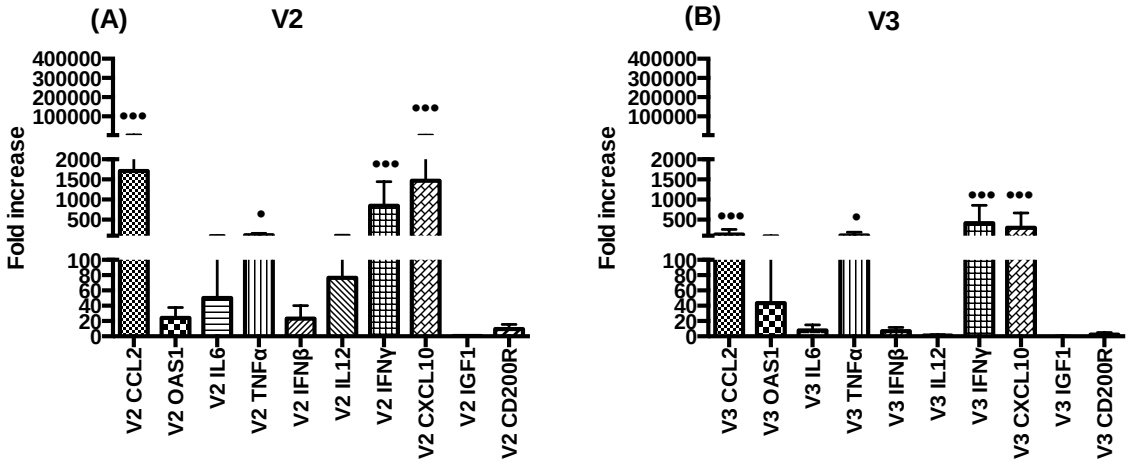

Figure 5. Relative gene expression of cytokines and chemokines in the brains of animals inoculated via i.c with V2 (A) or V3 (B) strains. Both variants showed increases in the following immune markers: IFNγ (***p<0.001), CXCL10 (***p<0.001), TNFα (*p<0.05) and CCL2 (***p<0.001).

CAPÍTULO 3

Artigo encaminhado para:

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Clinical and immune evaluation of mice experimentally infected with rabies virus variants 2 and 3 and treated with short-interfering RNAs (siRNAs)

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Abstract

We have evaluated the efficacy of siRNAs that target the nucleoprotein (N) gene and the brain immune response in infected mice and controls. Mice were inoculated with dog (hv2) or vampire bat (hv3) variants; for each variant a group was treated 24 h post virus infection (p.i.) and another was maintained as a control. Although no difference was observed in the lethality rate between the treated and non-treated groups, clinical evaluation of hv2 showed differences in the severity of clinical disease ($p = 0.0006$). A higher level of *N* gene expression at 10 versus 5 days p.i. was observed in the treated hv2 group ($p < 0.0001$). In the treated hv2 group, at 5 days no difference was found among the analysed genes, whereas at 10 days there was increased expression of 2',5'-oligoadenylate synthetase 1 (*OAS1*), tumor necrosis factor alpha (*TNF-α*), interleukin 12 (*IL12*), interferon gamma (*IFNγ*), and C-X-C motif chemokine 10 (*CXCL10*) relative to that in the hv2 non-treated group. Furthermore, higher *IFN-β* expression was found at 5 days in the hv2 non-treated group, but no difference was found in the hv3 siRNA-treated group. These results provide evidence of the efficacy of siRNA therapy in hv2 infection.

Keywords: rabies, dog virus, bat virus, treatment, siRNA

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1. Introduction

Rabies is a neglected zoonotic disease reported worldwide, except in Antartica. According to the World Health Organization (WHO), rabies is responsible for 55.000 deaths per year, with the great majority of deaths occurring in African and Asian countries. Most of the populations at risk live in poor rural areas and are mainly represented by children under 15 years of age [1, 2]. Rabies is classically caused by a rabies virus (RABV), a neurotropic, negative RNA single-stranded virus, of the genus *Lyssavirus* and in the family *Rhabdoviridae.* In theory, any rabid mammal can transmit rabies, usually through bites and scratches, but the bites of a rabid dog are responsible for 99.8% of the cases. In locations where dog rabies is controlled due to massive vaccination programs of domestic dogs and wild carnivores, such as in America, bats have become important in rabies transmission [1, 3, 4].

RABV causes acute encephalitis and rabies has a case-fatality rate approaching 100% being considered one of the most existent deadly infectious diseases [3]. The anti-rabies vaccine is very effective in preventing death and is a main pillar of the post-exposure prophylaxis (PEP) protocol, associated with wound cleaning and administration of rabies immunoglobulins. The efficacy of PEP depends on many factors like prompt intervention, adequate application of the immunoglobulin, the prescription of an adequate number of vaccine doses as well as the quality and right

preservation of the immune-biological. PEP seems to be ineffective after the onset of clinical signs, and death is almost a certain event [5, 6]. The survival of a 15-year-old girl from Wisconsin, bitten by a bat that received no vaccination, led physicians worldwide to apply the protocol known as the "Milwaukee Protocol" [7], but after 10 years it was shown to be ineffective. There are at least 26 reported cases in which this protocol was tested without success [5]. Therefore, continuous efforts should be made to find some effective treatment for rabies, including new technologies such as RNA interference.

RNA interference (RNAi) is an endogenous mechanism, first described in the late 90s that leads to post-transcriptional gene silencing. It is well conserved in a broad variety of species, including plants and animals [8, 9]. A short nucleotide sequence (aprox. 21–23 nucleotides length), also known as siRNA, associated with the RNA-induced silencing complex (RISC), recognizes and binds to a complementary mRNA, causing its cleavage into smaller fragments and inactivating its expression and, thus, inhibiting protein synthesis [10]. The RNAi mechanism plays an important role in cellular defense against viral infections in addition to other important cellular functions, including the mobility of genetic elements and regulation of gene expression during animal development [11]. The general potential of this mechanism has stimulated studies of the use of siRNA and microRNA as a therapeutic option for non-infectious [12, 13, 14] and infectious diseases, including dengue [15, 16], respiratory syncytial virus [17], influenza [18], tuberculosis [19], SARS [20], AIDS [13] and herpes simplex type 2 [21].

Despite the antiviral effect of siRNAs, they are potent activators of the mammalian innate immune system. Synthetic siRNA duplexes can induce high levels of inflammatory cytokines and type I interferons, after systemic administration in

mammals and in primary human blood cell cultures [22, 23]. The production of antiviral agents such as type I interferons, including interferon alpha (IFNα) and interferon beta (IFNβ), is an important immune mechanism against rabies virus infection that occurs soon after the cell infection [24, 25].

In 2007, Brandão and colleagues published a study in BHK-21 cells showing the efficacy of a novel therapy against rabies virus based on the use of siRNAs designed against the *N* gene sequence of Pasteur virus (PV). The results demonstrated that cells treated with three different sequences of siRNA had a five-fold drop in the amount of infected cells evaluated by direct immunofluorescence test when compared to controls, with any cytopathogenicity due to the treatment [26].

Studies testing siRNAs *in vitro* and *in vivo* usually have as the targets rabies nucleoprotein (N), glycoprotein (G) and/or polymerase (L) genes; the sequences are delivered by a vector such as adenovirus [27] or are associated with a liposome [28]. siRNAs always inhibit viral replication at some level, however it is difficult to precisely determine their real efficacy and possible application in medical practice. This is because in almost all studies, the siRNAs tested are those designed and checked in experimental infection due to the exact RABV strains (usually a laboratory strain) used as templates to design the siRNA sequences [11, 26, 27, 28].

This study aimed to test the clinical efficacy of three different sequences of siRNA designed against the RABV *N* gene in the treatment of mice infected with two different wild strains of RABV, isolated from rabid human patients infected by a dog or by a vampire bat variant. In addition, considering the difference of pathogenicity between dog and bat variants [29] and the immune stimulation that siRNA administration can induce, the brain immune response of infected and non-infected animals was evaluated.

2. Materials and methods

2.1 Experimental design

Two groups of 60 C57/BL6 mice, 4–6 week-old females, S.P.F, were inoculated in the gastrocnemius muscle with 100 µL of viral inoculum (LD_{50} $10^{-6.66}$/ 30 µL) variant 2 [dog (hv2)] or 3 [vampire bat (hv3)]. Half were treated intraperitoneally at 24-h p.i., with a unique dose of a mix constituted by three siRNA sequences (3.3 µM concentration each) designed against the *N* gene of the PV strain, using lipofectamine as the delivery method [26] (Table 1). Controls received sterile saline for intramuscular inoculation as well as for intraperitoneal treatment. A siRNA control group was included to evaluate possible side effects. For all groups, 10 animals were observed for 30 days and 10 were sacrificed after 5 and 10 days p.i., when whole brains were removed and stored at -80 ^{o}C until further real-time PCR analyses.

Animals of all groups were weighted and evaluated daily for the onset of clinical signs, such as ruffled fur, hunching back, hypo/hyper excitability, paralysis of one or both hind limb or tetraplegia [30].

The animal study was approved by the São Paulo State University Ethical Committee (registration number 238/2008), which follows the guidelines established by the COBEA- Brazilian Society of Laboratory Animals Science

2.2 RNA extraction and Real Time-RT-PCR (RT-qPCR)

Brain tissue RNAs were extracted with the Invitek® kit and stored at -80 °C. The reaction for cDNA synthesis consisted of 1 µg of extracted RNA, 1 µl of Oligo-DT primer (Invitrogen®) and 1 µl of SuperScript II (Invitrogen®) according to the manufacturer's instructions. The RT-qPCR reaction was performed with 2 µl of 1/50 diluted cDNA, 1 µl of 0.1 µg of each primer and Master Mix Syber Green (Promega®)

in a final volume of 25 µl according to the manufacturer's instructions. Primers for the 18S murine genes were supplied by IDT® and used as housingkeeping genes, and primers for the RABV *N* gene were manufactured as described previously [31]. The mouse Quantitect® Primer Assay from Qiagen® was used to evaluate the expression of chemokine C-C motif ligand 2 (CCL2), 2'-5'-oligoadenylate synthetase 1 (OAS1), interleukin 2 (IL2), interleukin 6 (IL6), interleukin 12 (IL12), tumor necrosis factor alpha (TNFα), interferon gamma (IFNγ), interferon beta (IFNβ), C-X-C motif chemokine 10 (CXCL10), cell surface glycoprotein CD200 receptor 1(CD200R) and insulin-like growth factor 1 (IGF-1).

All termal cycling and detection was performed using an Applied Biosystems StepOne Fast (ABI7500 Fast) thermal cycler employing a thermal profile of 40 cycles of 50 °C for 20 s, 95 °C for 10 min., 95 °C for 15 s and 60 °C for 1 min.

2.3 Data Analysis

Cox proportional hazards were used to estimate lethality rate and hazard ratios (HR) between groups. Kruskal-Wallis with $p < 0.05$ as the significance level was chosen for evaluation of the gene expression of cytokines/chemokines and the RABV *N* gene. Graph-Prism® 5.0 and Instat® softwares were employed as analysis tools.

3. Results and discussion

There was a nonsignificant statistical difference in mortality rate in the groups treated with siRNAs for any of the variants in this study. For hv2, the lethality was 100% in non-treated and 70% in treated groups ($p = 0.27$; HR = 0.57); for hv3, it was 60% in non-treated and 80% in treated groups ($p = 0.21$; HR = 1.97). (Fig. 1A).

Clinical evaluation of animals infected with variant 2 and treated with siRNA showed less severity of clinical disease, which included weight loss, paralysis, and

death ($p = 0.0006$) compared to the control. However, no clinical difference was observed for animals infected with hv3 and treated or non-treated with siRNA. (Fig. 1B).

The *N* gene expression of all groups compared at the same period, either at 5 or 10 days p.i., did not show any significant difference. However, *N* gene expression significantly increased in the hv2 treated group at 10 versus 5 days p.i. ($p < 0.0001$). The increase in *N* gene expression is expected, to follow the disease progression [30]. However biologically, hv2 siRNA-treated animals at 5 days had a very low expression compared to other groups at the same period, reflecting some interference of siRNA in virus replication in this study (Fig. 2).

The brain immune response of different groups showed a high expression of IFN-β ($p < 0.001$) at 5 days p.i. for the hv2 non-treated group and IFN-β ($p < 0.01$) and CCL2 ($p < 0.001$) for the hv3 non-treated group, and at 10 days p.i. the hv2 non-treated group showed increased IL12 expression ($p < 0.01$). There was no statistical difference in the immune markers analysed in the treated hv2 group at 5 days, whereas at 10 days OAS1, TNF-α, IL12, IFN-γ, and CXCL10 were increased ($p < 0.05$) (Fig. 3). Infected neurons showed a rapid production and release of IFN-β, which is important for host survival. This induces the expression of several IFN-stimulated genes (ISGs), such as *OAS1*, that exert an antiviral effect similar to IFN-β, but at different stages of viral replication [30]. Damaged neurons also produce IFN-γ, IL1, IL6, IL12, CCL2, CCL4, CCL5, CCL7 and the IFN-inducible protein, CXCL10. All these cytokines and chemokines are responsible for the upregulated expression of MHC molecules on the surface of microglia and also for the increased expression of adhesion molecules by endothelial cells. All the associated factors are important for induction of the adaptive immune response, which involves the activation and migration of T cells, as well as the

production of specific antibodies [32, 33, 34]. Enhanced *N* gene expression at 10 versus 5 days with hv2 indicates that the siRNA interfered with viral replication, which was not associated with a difference among cytokine/chemokine gene expression at 5 days (e.g., IFN-β), suggesting that viral levels were not sufficient to trigger the host immune response. This pattern of response did not occur in the hv2 non-treated group that showed a higher IFN-β expression at 5 days nor at hv3 treated group, which has a similar gene expression profile compared to the non-treated ones.

Considering the differences in the results between variant 2 and 3, the homology of siRNA sequences and the bat and dogs viruses employed in this study were blasted showing homology ranging from 95 to 100% for hv2 and 86 to 95% for hv3. The siRNAs tested were designed based on Paster virus (PV) [26], which is a fixed strain, and the homology results indeed showed a difference between street rabies virus and siRNA sequences, with the homology lower for the bat variant.

Although the *N* gene is considered a conserved site, studies have shown a significant genetic variability in street rabies virus strains, from 5 up to 49% [35, 36]. It is important to remember that almost a perfect complementary sequence between siRNA molecule and the viral RNA target is necessary to induce cleavage of mRNA [37]; this fact may explain the difference found in the results for dog and bat variants observed in this study.

To assess if the treatment with siRNA could be at least partially effective in the symptomatic phase of the disease, a similar study included mice that received a single dose of siRNAs only after the onset of clinical signs. No difference in the mortality rate, clinical evaluation or prolongation in the evolution period was observed in any of the treated groups (data not shown).

The immune response evaluation in non-inoculated animals treated with siRNA showed an up-regulation of all immune markers, in particular that of IFN-γ at 5 days compared to 10 days. This increase disappeared at 10 days, reflecting the reduction in the activity of siRNA, which can last up to 6 days [38] (Fig. 4).

4. Conclusion

Therapy with siRNA did not reduce the lethality rate in two different street rabies virus infections. However, there were less severe clinical signs using siRNA therapy in variant 2 infection. The efficacy of siRNA therapy is closely associated with the homology of the siRNA design and its target. In this study, a higher homology, no doubt, was reached with variant 2 despite variant 3, justifying the results obtained. A reduced expression of immune markers with siRNA therapy in infected, but not in non-infected mice, may occur as a result of the antiviral effect. These data provide evidence of some efficacy of siRNA therapy in rabies virus infection due to a wild-type dog strain. However, it is clear that the potential in applying this technology is limited because in medical practice the type of virus infecting the patient is unknown in almost 100% of the cases. More studies are necessary to overcome this problem and to show this technology could be applied either alone or associated with other therapeutic measures.

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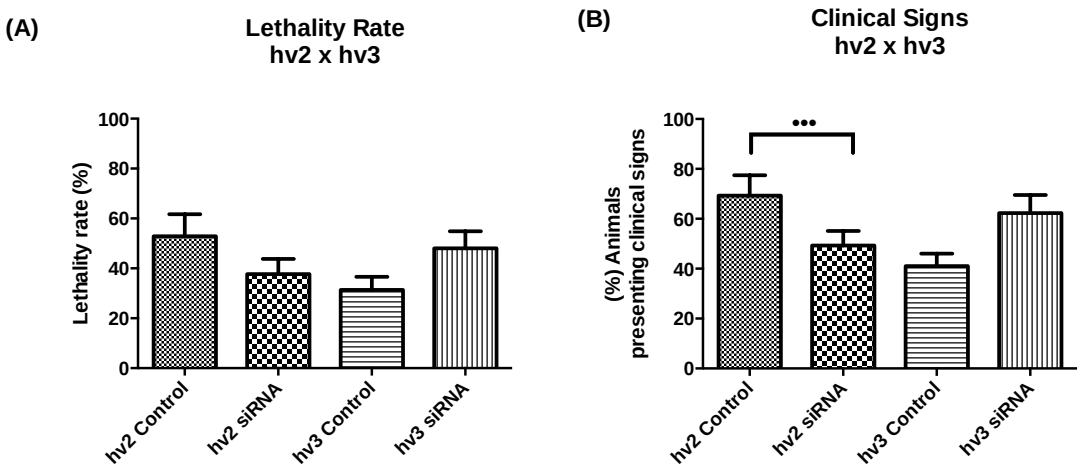

Figure 1. (A) Lethality rate of controls and siRNA-treated groups inoculated with variant 2 (hv2) and variant 3 (hv3); Cox proportional hazards were used to estimate lethality rates and hazard ratios between groups. No statistical difference was found. (B) Percentage of animals in each group showing clinical signs, which included weight loss, ruffled fur, hunched back, hypoexcitability, hyperexcitability, paralysis and tetraplegia; Kruskal-Wallis test showed a statistical difference ($p = 0.0006$) between the hv2 control and siRNA-treated group.

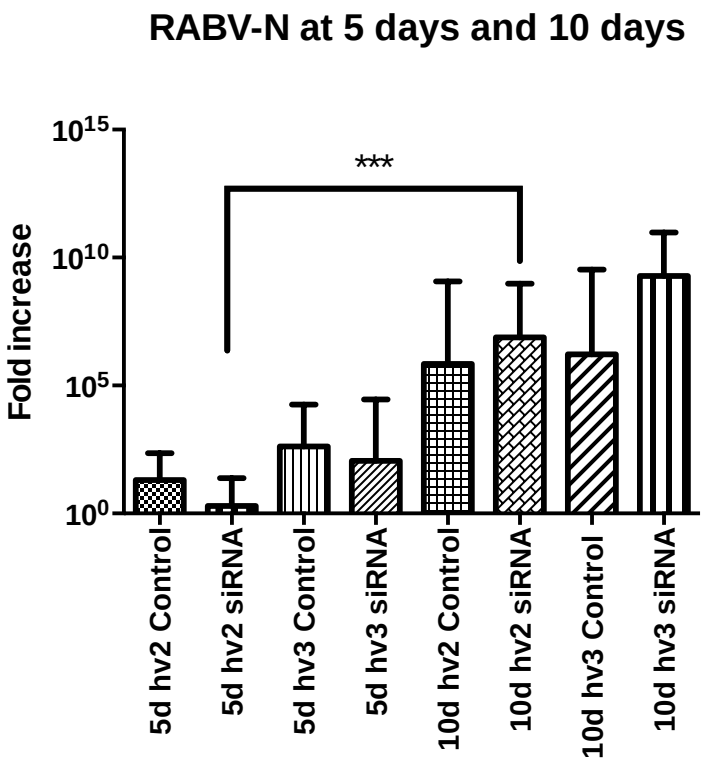

Figure 2. RABV *N* gene expression in the brain of mice infected with hv2 or hv3. Kruskal-Wallis test to compare the results between different groups at 5 and 10 days p.i. There was no difference between the hv2 and hv3 groups at 5 or at 10 days. However, hv2 siRNA-treated groups showed a significant difference ($p < 0.0001$) at 5 versus 10 days.

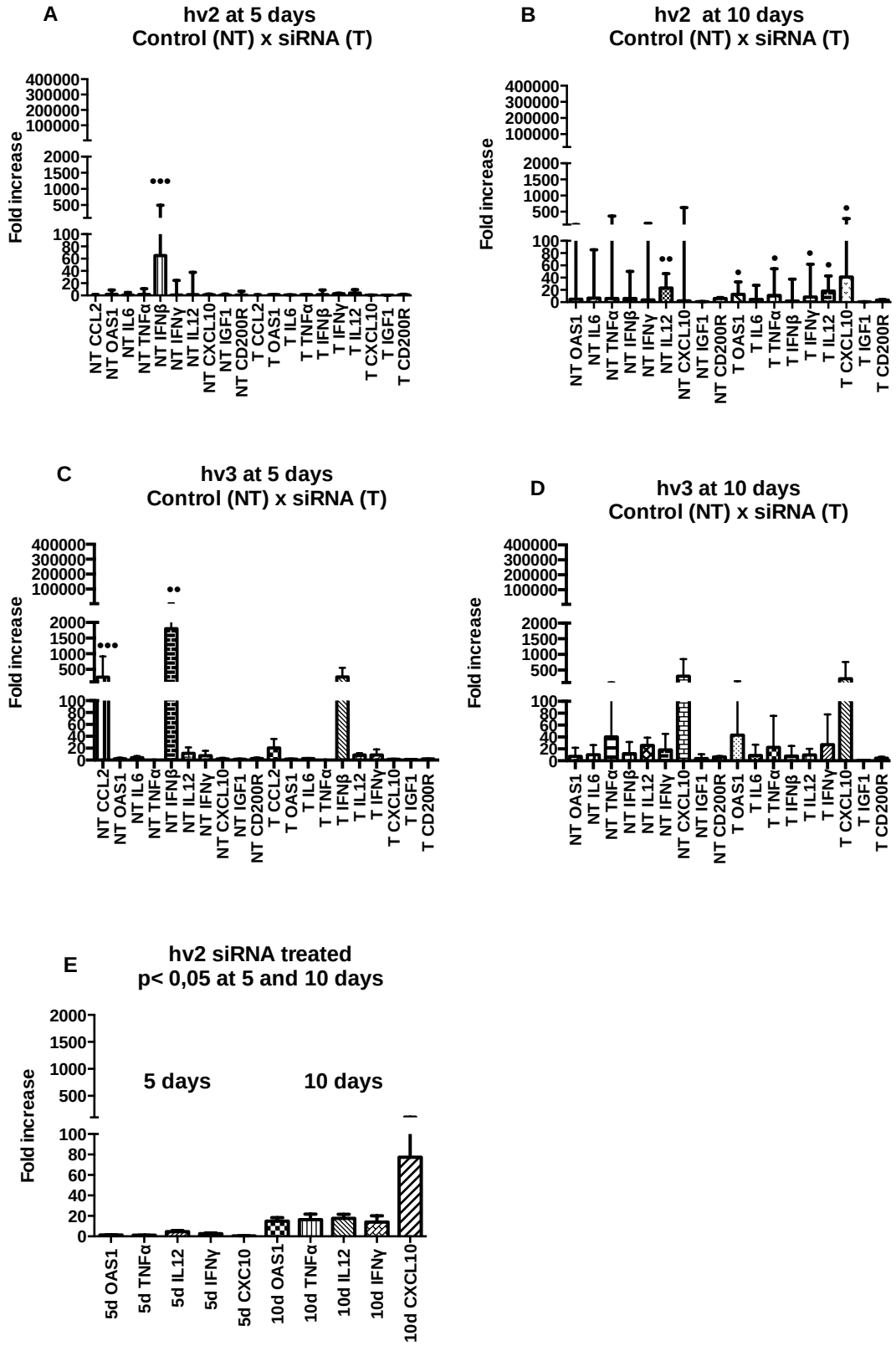

Figure 3. Relative gene expression of cytokines and chemokines in different groups (hv2 and hv3) at 5 and 10 days p.i. The Kruskal-Wallis test was applied to analyse the results. (A) Expression at 5 days p.i. in the hv2 control (NT) and siRNA-treated (T)

groups; IFNβ was highly expressed in the NT group ($^{***}p < 0.001$). (B) Expression at 10 days p.i. in the hv2 control (NT) and siRNA-treated (T) groups; IL12 was highly expressed in the NT group ($^{**}p < 0.01$); in the treated group, *OAS1*, *TNFα*, *IL12*, *IFNγ* and *CXCL10* expression levels were increased ($^{*}p < 0.05$). (C) Expression at 5 days p.i in the hv3 control (NT) and siRNA-treated (T) groups; IFNβ was highly expressed in the NT group ($^{**}p < 0.01$). (D) Expression at 10 days p.i. in the hv3 control (NT) and siRNA-treated (T) groups; no significant difference was found. (E) Only statistically different cytokines and chemokines at 5 versus 10 days p.i. in the hv2 siRNA-treated group, *OAS1*, *TNFα*, *IL12*, *IFNγ* and *CXCL10* were increased at 10 days p.i. ($^{*}p < 0.05$).

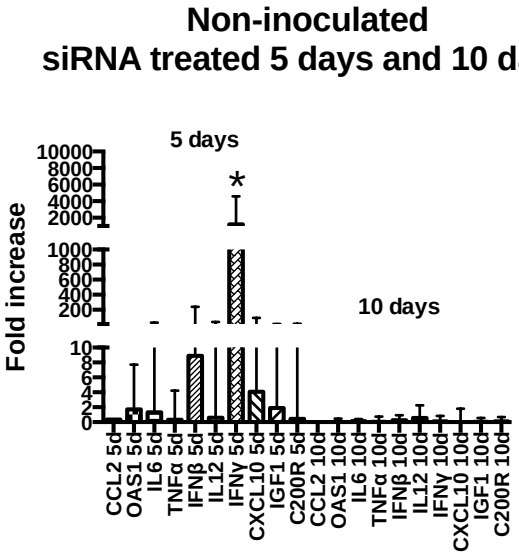

Figure 4. Relative expression of immune markers at 5 and 10 days in mice brains after administration of siRNAs. The treatment led to an increase in the gene expression of *IFNγ* ($^{*}p < 0.05$) at 5 days. The Kruskal-Wallis test was used to compare the results in different groups.

Table 1. Nucleotide sequences of siRNAs designed by Brandão (2007) [26] against PV *N* gene.

siRNA	Douplex Sequence
RNA124	sense 5'GCCUGAGAUUAUCGUGGAG 3' antisense 5'AUCCACGAUAAUCUCAGGC 3'
RNA750	sense 5'GCACAGUUGUCACUGCUUC3' antisense 5'UAAGCAGUGACAACUGUGC 3'
RNA B	sense 5'GACAGCUGUUCCUCACUCG 3' antisense 5'AGAGUGAGGAACAGCUGUC 3'

CAPÍTULO 4

Discussão Geral

Resposta imune nos animais inoculados por via intramuscular com variante 2 e 3

Embora a análise da sobrevivência tenha sido estatisticamente distinta entre os grupos, nenhuma diferença foi observada quanto ao período de incubação e evolução nas diferentes variantes. Um estudo recente de casos humanos, comparando as principais características da enfermidade causada por um virus de cão ou um virus de morcego, constatou que após o inicio da sintomatologia clinica, nenhuma diferença no tempo de sobrevida foi observada comparando as duas variantes (UDOW et al., 2013).

A expressão do gene *N* foi maior aos 10 dias comparativamente aos 5 dias sem diferença estatística quando considerados os vários grupos e periodos avaliados. No entanto, quando comparamos a expressão entre 5 versus 10 dias dentro de cada grupo, aquele inoculado com a maior DL_{50} da variante 2 apresentou um aumento significativo aos 10 dias, o que poderia também estar associado a maior taxa de letalidade apresentada neste grupo. Contrariamente a estes resultados, em um estudo publicado por Chopy et al. (2011), camundongos inoculados por via intramuscular com uma dose letal de CVS foram sacrificados em diferentes estágios clínicos da doença, não sendo observadas diferenças na expressão de *N*. A diferença entre o estudo atual e o publicado, pode ser justificada pela natureza das amostras, fixa ou de rua.

Comparando a variante 2 (V2) e 3 (V3) com mesma DL_{50} e mesmo resultado de letalidade, o perfil de citocinas e quimiocinas sugerem que V2 tem capacidade de induzir a expressão de um maior número de genes, e com maior intensidade de expressão, especialmente aos 10 dias quando comparado a V3. Estudo feito em camundongos inoculados com mesma dose letal, por via intracerebral ou intramuscular, com amostra altamente patogênica (SHBRV) ou com amostra atenuada (B2C) demonstrou que a quantidade de genes supra-

regulados pela B2C eram em maior número e intensidade quando comparados a SHBRV (WANG et al., 2005). Apesar de ambas as amostras virais utilizadas tenham sido classificadas como altamente patogênicas, pois foram isoladas de pacientes humanos com raiva, a expressão gênica de V2 e V3 com mesmo título viral e mesmo percentual de letalidade, foram biologicamente diferentes, especialmente aos 10 dias, quando V2 induziu a uma maior expressão quando comparado a V3, demonstrando que a letalidade não parece estar associada com a expressão gênica no presente estudo.

Comparando-se a variante 2 adminstrada em diferentes DL_{50}, com taxa de letalidade distinta entre os grupos, aos 5 dias, o grupo inoculado com $40DL_{50}$ apresentou expressão mais elevada de IL12, e no grupo inoculado com $80DL_{50}$, maior expressão de IFNβ, sendo que esta ultima, somente apresentou-se elevada no grupo inoculado com 40 DL_{50} aos 10 dias. Estudos feitos por Chopy et al. (2011), avaliando a capacidade da amostra CVS de RABV em desencadear e evadir a resposta de IFN tipo I em células, mostrou que quanto maior a multiplicidade de infecção (MOI), mais precoce a expressão de IFNβ, no entanto a magnitude da resposta não foi determinada pela quantidade de virus. Este resultado explica, ao menos em parte, a razão pela qual no presente estudo, o grupo inoculado com 80 DL_{50} teve uma expressão precoce IFNβ. A expressão do gene *N* aos 5 dias, no grupo inoculado com 80 DL_{50} foi duas vezes menor quando comparado ao grupo inoculado com 50 DL_{50}, no entanto, esta diferença despareceu aos 10 dias, sugerindo que a ação antiviral de IFNβ no grupo inoculado com 80 DL_{50} foi bastante eficiente em conter a replicação viral, no entanto, assim que os mecanismos de evasão viral foram desencadeados, a expressão de IFNβ caiu e os níveis do gene *N* aumentaram aos 10 dias (LAFON, 2008; LAFON, 2011). Mesmo havendo um aumento de IFNβ aos 10 dias no grupo inoculado com 40 DL_{50}, este não foi mais eficaz em conter a replicação viral, uma vez que um determinado número de células já estavam infectadas (MASATANI et al., 2013), sendo isto comprovado pelo fato do gene *N* estar igualmente elevado em ambos os grupos.

Interessante notar que, embora a expressão de OAS1, bastante elevada aos 5 dias no grupo inoculado com 40 DL_{50}, seja induzida pela presença de IFNβ e também exerça uma atividade antiviral (GRIFFIN, 2003), esta citocina não foi suficiente para inibir a replicação de RABV tanto quanto IFNβ, demonstrando que talvez o IFNβ seja mais efetivo, e portanto, a primeira citocina a ser produzida na fase inicial das infecções. A expressão elevada de IL-12 no grupo inoculado com 40 DL_{50}, sugere que uma menor dose viral foi capaz de induzir uma resposta imune mediada por células de uma maneira mais precoce, uma vez que esta citocina é liberada por macrófagos, células dendríticas e células NK ativadas durante o processo de infecção, o que também explicaria a ativação de uma grande quantidade de genes aos 10 dias, assim como a menor letalidade observada neste grupo. Contrariamente, o grupo inoculado com 80 DL_{50}, apresentou uma marcante resposta antiviral aos 5 dias, porém sem sinal de ativação precoce dos outros componentes da resposta imune celular, evidenciada pela baixíssima expressão dos marcadores da resposta aos 10 dias. Estes resultados sugerem a importância da imunidade inata na sobrevivência da raiva (GRIFFIN, 2003; LI et al., 2008; ZHAO et al., 2010).

Resposta imune nos animais inoculados por via intracerebral com a variante 2 e 3

Nos animais avaliados em fase terminal da enfermidade, inoculados com V2 ou V3, embora sem diferença no percentual de letalidade, a analise da curva de sobrevivência demostrou diferença na progressão da doença em ambas; o perido de incubação, mais longo para V3 e o de evolução, mais longo para V2. Um estudo conduzido na Índia que analisou dados de pacientes humanos de raiva, mostrou que o período de incubação não estava correlacionado com a rapidez do curso clínico da doença (SOLANKI et al., 2009). O curto período de evolução em V3 pode ser positivamente associado com a elevada expressão do gene *N* deste grupo.

A avaliação da resposta imune em ambos os grupos demonstrou aumento das mesmas citocinas e quimiocinas, sendo elas IFNγ, CXCL10, TNFα e CCL2. A principal atividade de quimiocinas como CXCL10 e CCL2 é modular o tráfico de células T para o SNC, determinante para a eliminação viral. Estudos realizados com herpes simplex e West Nile virus mostraram que a deleção do gene responsável pela expressão CXCL10 levou a uma fraca infiltração de células T, bem como uma baixa taxa de eliminação viral que foi acompanhada por um quadro clinico grave, no entanto, também existem evidencias de que o excesso de células T possa ser deletério, pois promove maior citotoxicidade e inflamação severa (ZHAO et al., 2009; NIU et al., 2011). Estudos *in vivo* ou *in vitro* demonstraram que a expressão de CXCL10 está presente e supra-regulada após infecção por RABV (WANG et al., 2005; MANSFIELD et al., 2008; ZHAO et al., 2013). Esta quimiocina induz aumento da permeabilidade da BHE, o que parece essencial para o *clearance* de RABV do SNC, pois permite a migração de células efetoras e também de anticorpos (KUANG et al., 2009). Os estudos realizados por Wang et al. (2005) e Kuang et al. (2009) descreveram um aumento na expressão de CXCL10 associado a alta expressão de TNFα em camundongos infectados com amostras atenuadas de RABV, mas esta supra-regulação não foi evidenciada nas infecções por virus de rua, o que difere dos resultados obtidos no presente estudo. Em estudo conduzido por Solanki et al. (2009), em cortes histológicos de cérebros positivos para raiva, uma alta de expressão de TNFα e IL1, que são citocinas pró-inflamatórias, foi observada, no entanto, este aumento não estava correlacionado a quantidade de virus detectada no local.

Comparando os valores da expressão das citocinas e quimiocinas entre V2 e V3, a expressão de CCL2 e CXCL10 foram maiores em V2, comparativamente a V3. A elevada expressão destas quimiocinas está relacionada a atração de células T e ao aumento de permeabilidade da BHE, e neste presente estudo, possivelmente, justifica o menor nível na expressão do gene *N* neste grupo não estando, no entanto, associado a uma diminuição na taxa de letalidade, uma vez que ambas foram de 100%. MANSFIELD et al. (2008) observaram uma elevada expressão de

CXCL10, IL6 e IFNγ em camundongos inoculados com amostra de rua, altamente patogênica, o que no entanto, não garantiu a sobrevivência dos animais. Pode-se concluir, a partir dos resultados deste estudo, que embora uma resposta imune robusta tenha ocorrido nos animais inoculados com ambas as variantes, esta não foi suficiente para controlar a infecção e impedir a morte dos animais, demonstrando que mais além do perfil da resposta, a precocidade com que ela surge pode ser crucial.

Terapia com siRNA

Embora nenhuma diferença estatística tenha sido notada na variante 2 ou 3, quanto ao percentual de letalidade entre os grupos tratados e não-tratados, uma menor severidade clinica, que incluía perda de peso, paralisia e morte, foi observada nos animais tratados e inoculados com V2, o mesmo não sendo observado para o grupo inoculado com V3.

A expressão do gene *N* não variou entre os grupos aos 5 ou 10 dias após a inoculação, no entanto, no grupo tratado e inoculado com V2, *N* aumentou siginificativamente aos 10 versus 5 dias. O aumento da expressão de *N* é um evento esperado ao longo da evolução da doença, mas os animais tratados com siRNA pertencentes ao grupo que havia sido inoculado com V2 tiveram uma expressão muito menor de *N* aos 5 dias em relação aos outros grupos no mesmo período, refletindo alguma interferência do siRNA na replicação viral neste estudo.

A possibilidade de interferência do siRNA na replicação viral também ficou evidenciada na resposta imune, pois enquanto aos 5 dias, o grupo de animais não-tratados apresentou uma alta expressão de IFNβ e CCL2, nenhuma diferença estatística foi observada no grupo de animais tratados e inoculados com V2. Aos 10 dias, V2 não-tratado apresentou apenas aumento na expressão de IL12, enquanto o grupo tratado e inoculado com a mesma variante, apresentou expressão aumentada de OAS1, TNFα, IL12 , IFNγ, e CXCL10, sendo estas citocinas e quimiocinas importantes pois exercem atividade antiviral,

atuam como mediadoras da resposta imune especifica e também levam ao aumento de permeabilidade da BHE (GRIFFIN, 2003; KOYAMA et al., 2008; CHOPY et al., 2011; STEEL et al., 2014) . A baixa expressão aos 5 dias, de citocinas e quimiocinas no grupo tratado inoculado com V2 , seguida de um aumento destas aos 10 dias neste mesmo grupo, associado também a um perfil similar na expressão do gene *N*, sugerem que aos 5 dias, a quantidade de virus presente no tecido não foi suficiente para o desencadeamento da resposta imune, como ocorreu nos demais grupos, no entanto, assim que a atividade de siRNA começou a diminuir, em torno de 6 dias após o tratamento (CHIU e RANA, 2003), RABV voltou a se replicar o que consequentemente induziu uma resposta, como observado aos 10 dias.

Considerando-se os diferentes resultados entre as variantes 2 e 3, a homologia entre as sequencias de siRNA e o gene *N* das variantes de cão e de morcego utilizadas neste estudo foram analisadas, e revelou maior compatibilidade com a variante 2 (95-100%) do que com a variante 3 (86-95%). Os siRNAs testados neste estudo foram desenvolvidos, utilizando como base, a sequencia do gene *N* da cepa Pasteur, que é uma amostra fixa derivada de cão, o que justifica a maior homologia com esta variante, comparativamente a variante de morcego.

Embora o gene *N* seja considerado um dos mais conservados no virus da raiva, estudos revelam uma grande variabilidade genética nas amostras de rua, em torno de 5 a 49% (DENDUANGBORIPANT et al., 2005, ISRASENA et al., 2009). É importante salientar que uma complementariedade perfeita é necessária entre o siRNA e a sequencia-alvo para que a clivagem do RNAm ocorra (ISRASENA et al., 2011), este fato pode explicar os diferentes resultados obtidos neste estudo para as variantes de cão e de morcego.

Conclusões Gerais

No presente trabalho um mecanismo de evasão viral mais eficiente foi observado no grupo infectado com a DL_{50} mais elevada (80V2).

O mecanismo de evasão foi caracterizado pela inibição de IFNβ, com conseqüente diminuição da expressão das citocinas e quimiocinas analisadas, associada a uma alta expressão do gene *N* de RABV.

As variantes 2 e 3 inoculados com mesma DL_{50}, por via intramuscular, apresentaram mesmo percentual de letalidade e aspectos clínicos, mas diferiram quanto ao perfil de expressão gênica, indicando que a estimulação da resposta imune é intrínseca à variante de RABV.

A produção precoce de citocinas e quimiocinas, parece ser mais importante do que os seus níveis de expressão para a sobrevivência na raiva, como observado nos animais agônicos, que embora expressando níveis elevados dos genes relacionados a resposta imune específica, sucumbiram à doença.

A terapia com siRNA não reduziu a taxa de letalidade nos animais inoculados com as diferentes variantes virais, no entanto, os sinais clínicos foram menos severos nos animais inoculados com a variante 2 e tratados com siRNA, quando comparado aos animais do grupo controle.

A eficácia da terapia de siRNA está intimamente associada com a homologia complementar da sequencia de siRNA e sua sequencia - alvo.

Os dados obtidos neste estudo fornecem evidência de interferencia do siRNA na replicação da amostra de rua do vírus da raiva, variante 2, admistrada 24 horas após a inoculação, no entanto, sem qualquer efetividade quando administrada na fase clinica da enfermidade.

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Veterinary Immunology and Immunopathology

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List of standard abbreviations:

Ab	Antibody
Ag	Antigen
AIDS	Acquired immunodeficiency syndrome
APC	Antigen-presenting cell
BCG	Bacillus Calmette Guerin
BSA	Bovine serum albumin
C	Complement
CFU	Colony-forming unit
ConA	Concanavalin A
cpm	Counts per minute
CSF	Colony-stimulating factor
CTL	Cytotoxic T lymphoctye
DMSO	Dimethylsulfoxide
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme-linked Immunosorbent assay
Fab	Monovalent antigen-binding fragment
F(ab1)2	Divalent antigen-binding fragment
FBS	Fetal bovine serum
FCS	Fetal calf serum
Fc&Ggr;R	Receptor for the Fc&Ggr; part of the IgG
FITC	Fluorescein Isothiocyanate
H chain	Heavy chain of Ig
HIV	Human immunodeficiency virus
HLA	Human histocompatibility leukocyte antigen
IFN	Interferon
Ig	Immunoglobulin
IL	Interleukin
i.d.	Intradermal
i.m.	Intramuscular
i.p.	Intraperitoneal
IU	International unit
i.v.	Intravenous

KDD Kilodalton (mol. mass)

KLH Keyhole limpet hemocyanin

L chain Light chain of Ig

LAK Lymphokine-activated killer (cell)

LPS Lipopolysaccharide

mAb Monoclonal antibody

MHC Major histocompatibility complex

MLR Mixed lymphocyte (leukocyte) reaction

M&Fgr; Macrophage

Mr Relative molecular mass

n Number in study or group

ND Not determined

NK Natural killer (cell)

OD Optical density (transmission through turbid suspensions)

OVA Ovalbumin

p Probability

PAGE Polyacrylamide gel electrophoresis

PBL Peripheral blood lymphocytes

PBMC Peripheral blood mononucleur cells

PCR Polymerase chain recation

PBS Phosphate-buffered saline

PHA Phytohemagglutinin

PKC Protein kinase C

PMA Phorbol 12-myristate 13-acetate

PMN Polymorphonuclear (cell, leukocyte)

PPD Purified protein derivative of tuberculin

PWM Pokeweed mitogen

r Recombinant

R Receptor (e.g. IL-2R)

RBC Red blood cells

RFLP Restriction fragment length polymorphism

RIA Radioimmunoassay

RNase Ribonuclease

s.c. Subcutaneous

SD Standard deviation
SDS Sodium dodecyl sulfate
SEM Standard error of the mean
SRBC Sheep red blood cells
SV40 Simian virus 40
TCR T cell receptor
Th T helper (cell)
TGF Transforming growth factor
TNF Tumor necrosis factor
Ts T suppressor (cell)
U Unit
V region Variable region
1°, 2°, 3° Primary, secondary, tertiary
2-D Two dimensional
3-D Three dimensional

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