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Evaluation of short-interfering RNAs treatment in experimental rabies due to wild-type virus

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ABSTRACT

We have evaluated the efficacy of short-interfering RNAs targeting the nucleoprotein gene and also the brain immune response in treated and non-treated infected mice. Mice were inoculated with wild-type virus, classified as dog (hv2) or vampire bat (hv3) variants and both groups were treated or leaved as controls. No difference was observed in the lethality rate between treated and non-treated groups, although clinical evaluation of hv2 infected mice showed differences in the severity of clinical disease ($p = 0.0006$). Evaluation of brain immune response 5 days post-inoculation in treated hv2 group showed no difference among the analyzed genes, whereas after 10 days post-inoculation there was increased expression of 2′,5′-oligoadenylate synthetase 1, tumor necrosis factor alpha, interleukin 12, interferon gamma, and C-X-C motif chemokine 10 associated with higher expression of *N* gene in the same period ($p < 0.0001$). In hv2 non-treated group only higher interferon beta expression was found at day 5. The observed differences in results of the immune response genes between treated and non-treated groups is not promising as they had neither impact on mortality nor even a reduction in the expression of *N* gene in siRNA treated animals. This finding suggests that the use of pre-designed siRNA alone may not be useful in rabies treatment.

Introduction

Rabies virus (RABV) causes acute encephalitis and has a case-fatality rate approaching 100% being considered one of the most deadly existent infectious diseases.[1] 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"[2] but after 10 years it has been shown to be ineffective. There are at least 26 reported cases in which this protocol was tested without success.[3] Therefore, continuous efforts should be made to find some effective treatment for rabies, including new technologies such as RNA interference.

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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.[4,5] A short nucleotide sequence (approx. 21–23 nucleotides length), also known as short-interfering RNA (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.[6] 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.[7] The general potential of this mechanism has stimulated studies of the use of siRNA and microRNA as a therapeutic option for non-infectious[8–10] and infectious diseases, including dengue,[11,12] respiratory syncytial virus,[13] influenza,[14] tuberculosis,[15] SARS,[16] AIDS,[10] and herpes simplex type 2.[17]

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.[18,19] 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.[20,21]

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 no cytopathogenicity due to the treatment.[22] Those same sequences were tested by the same group *in vivo* and demonstrated reduction in the lethality rate when compared to untreated animals.[23]

Studies testing siRNAs *in vitro* and *in vivo* usually have as targets rabies nucleoprotein (N), glycoprotein (G), and/or polymerase (L) genes; the sequences are delivered by a vector such as adenovirus,[24] lentivirus,[25] or associated with a liposome.[26] 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 exactly the same RABV strains (usually a laboratory strain) used as templates to design the siRNA sequences.[7,22,24,26]

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[27] and the immune stimulation that siRNA administration can induce, the brain immune response of infected and non-infected animals was evaluated.

Table 1 – Nucleotide sequences of siRNAs designed against Pasteur virus *N* gene.[22]

siRNA	Duplex 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′

Materials and methods

Experimental design

Two groups of 60 C57/BL6 mice each, 4–6 week-old females, S.P.F, were inoculated in the gastrocnemius muscle with 100 μL of viral inoculum with same viral titration (LD_{50} $10^{-6.66}$/30 μL) for variant 2 [dog (hv2)] or variant 3 [vampire bat (hv3)]. Thirty animals were treated intraperitoneally 24 h p.i., with a unique dose of a mixture consisting of three siRNA sequences (3.3 μM concentration each) designed against the *N* gene of the PV strain, using lipofectamine as the delivery method[22] (Table 1); the other half ($n = 30$) were left untreated, and just received saline intraperitoneally at the same time of the siRNA treated group. A non-inoculated group of animals ($n = 30$) were used as controls for basal immune response and received intramuscular of sterile saline as well as intraperitoneal inoculation. A non-inoculated siRNA treated group ($n = 30$) was included to evaluate possible side effects of the treatment and also the immune stimulation of siRNA. For all groups, 10 animals were observed for 30 days and 10 were euthanized after 5 and 10 days p.i., when whole brains were removed and stored at −80 °C until further real-time PCR analyses.

Animals of all groups were weighted and evaluated daily for the onset of rabies clinical signs, such as ruffled fur, hunching back, hypo/hyper excitability, paralysis of one or both hind limb or tetraplegia,[28] and for any other abnormality in the case of the non-inoculated groups.

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 College of Animal Experimentation).

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 housekeeping genes, and primers for the RABV *N* gene were manufactured as described previously.[29] The mouse