APCRI Journal
Volume 25, Issue 2 - 2023, Pg. No. 1-7
Peer Reviewed & Open Access Journal

Research Article

Virus Hybrid Vectored Particles Carrying an RNA Coding for Rabies Virus Glycoprotein

Thaissa Consoni Bernardino[1], Sandra Fernanda Patiño Suarez[1], Carlos Augusto Pereira[1,+], Renato Mancini Astray[2], Ana Sofia Coroadinha[3], Hugo Soares[3], Soraia Attie Calil Jorge[1]

[1]Viral Immunology Laboratory, Butantan Institute, Sao Paulo-SP, Brazil.
[2]Multipurpose Laboratory, Butantan Institute, Sao Paulo-SP, Brazil.
[3]Cell Line Development and Molecular Biotechnology Laboratory, Institute of Experimental and Technological Biology, Oeiras, Portugal.
[+]In memoriam
DOI: https://doi.org/10.24321/0973.5038.202308

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Corresponding Author:
Thaissa Consoni Bernardino, Viral Immunology Laboratory, Butantan Institute, Sao Paulo-SP, Brazil.
E-mail Id:
thaissa.bernardino@fundacaobutantan.org.br
Orcid Id:
https://orcid.org/0000-0001-8173-1793
How to cite this article:
Bernardino TC, Suarez SFP, Pereira CA, Astray RM, Coroadinha AS, Soares H, Jorge SAC. Virus Hybrid Vectored Particles Carrying an RNA Coding for Rabies Virus Glycoprotein. APCRI J. 2023;25(2):1-7.

Date of Submission: 2023-05-25
Date of Acceptance: 2023-07-09

A B S T R A C T

Introduction: This study was aimed at establishing a system for producing virus hybrid vectored particles (VHVP) capable of carrying the RNA of the rabies virus glycoprotein (VHVP-RVGP).

Method: To produce VHVP-RVGP, HEK 293T cells were co-transfected with pCMV-Gag-Pol (from leukemia murine virus), pCMV-E1E2 (glycoproteins of hepatitis C virus), and pCMV-RVGP. VHVP-RVGP were collected 48 hours post-co-transfection and titrated by quantitative RT-PCR (RTq-PCR). The RVGP expression was detected by ELISA in lysates of Huh-7 cells treated with VHVP-RVGP.

Results: RT-qPCR assays that were conducted for the detection of cell attachment, as well as entry of VHVP-RVGP, revealed that only a small part was able to enter Huh-7 cells, indicating a limitation in the transduction process. Western blotting assays showed that MLV's Gag-Pol protein production as well as the incorporation of HCV E1E2 into VHVP were successful.

Conclusion: In conclusion, the data demonstrated that the VHVP system was efficient in carrying the RNA-RVGP, although the HCV E1 and E2 incorporation was not efficiently presented for a proper VHVP conformation.

Keywords: Rabies Virus Glycoprotein, Virus Hybrid Vectored Particles, Quantitative RT-PCR, Rabies Vaccine

Introduction

Despite the willingness to create effective and safe vaccines for human and animal protection, rabies, a disease transmitted by domestic/ wild animals, is still widespread all over the world. The number of human deaths due to rabies has been estimated at 60,000 annually, most of them being in Africa and Asia.[1]

The transmembrane rabies virus glycoprotein (RVGP) is the main rabies virus antigen that can trigger an immunological response.[2] To induce an immune response, RVGP depends on complex post-translational modifications.[3]

Different expression systems have been used to express the rabies glycoprotein, such as yeast,[4] BHK 21 mammalian cells,[5] and Sf9 insect cells using the baculovirus system,[6] among others. In the past years, new technologies of gene expression have emerged,[7] such as the virus hybrid vectored particles (VHVP), a system derived from hepatitis C virus pseudoparticles (HCVpp) described by Bartosch et al.[8] The HCVpp system was designed to investigate the role of HCV E1 and E2 glycoproteins along with their possible receptors in cell entry, the host range of HCV, and antibody neutralization from HCV patient sera. This was necessary due to the lack of an effective culture system capable of amplifying the virus, which hindered the development of reliable infection assays.[8]

The polyproteins of the murine leukemia virus (Gag-Pol) were used in the present study. They have the capacity to form vectored particles and incorporate membrane proteins, like E1 and E2 of the hepatitis C virus (HCVpp).[8,9] However, there is a lack of data on heterologous protein expression. This study used the RVGP gene to monitor for heterologous RNA transduction. This optimized system may be useful for RNA vaccines in target cell delivery. Therefore, this study was performed to analyze the production of virus hybrid vectored particles capable of packing and carrying the RNA of RVGP (VHVP-RVGP) as a possible use for vaccination.

Material and Methods

Vectors

The vectors pCMV-GFP, pCMV-E1E2, and pCMV-Gag-Pol were kindly granted by Dr François-Loïc Cosset, INSERM, France. pCMV-GFP was used to construct the pCMV-RVGP.

Cell Culture

HEK 293T (human embryo kidney) and Huh-7 (human hepatocellular carcinoma) cells were first cultured in high-glucose Dulbecco's modified Eagle's medium (DMEM) (Life Technologies, Glasgow, UK) containing 10% (v/v) of FBS (Sigma, St. Louis, USA) under 5% CO_2 atmosphere at 37 °C.

Co-transfection Assays

To generate virus hybrid vectored particles that carry the RNA of the rabies virus glycoprotein, HEK 293T cells were co-transfected with expression vectors encoding the viral components using Lipofectamine® 2000 Transfection Reagent (Thermo Fisher Scientific™) in six well plates/ 25 cm^2 t-flasks/ petri plates according to the manufacturer's protocol (Figure 1).

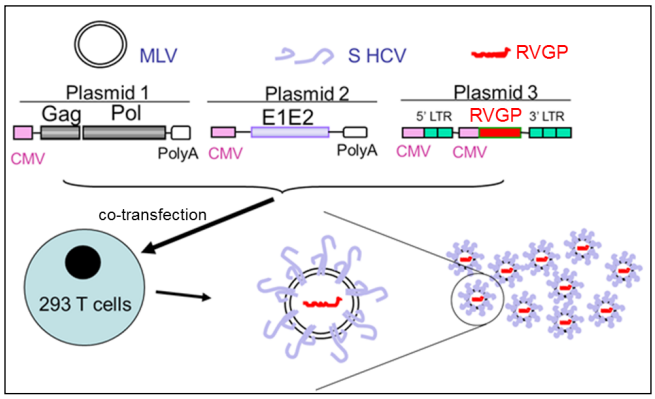

Figure 1.Vectors Encoding the Various Components Needed to Assemble Infectious Virus Hybrid Vectored Particles. CMV represents cytomegalovirus immediate-early promoter; LTR refers to long terminal repeat; MLV represents structural proteins of MLV; the S HCV represents structural proteins of HCV; RVGP represents the rabies virus glycoprotein gene; and Poly A refers to the polyadenylation site.

The amounts used were as follows: 2.7 µg of pCMVE1E2, 8 µg of pCMV-Gag-Pol, 8.1 µg of pCMVRVGP, and 20 µl of lipofectamine. 48 hours post-co-transfection, the supernatant was collected, and stored at -80 °C till cell transduction or quantification of VHVP. Some viral batches were concentrated with Vivaspin 2 (30 kDa molecular weight (GE Healthcare®, Uppsala, Sweden)).

RNA Extraction and cDNA Synthesis

RNA-RVGP was isolated from VHVP-RVGP produced in HEK 293T cells. It was used in QIAamp Viral RNA Mini-Kit (QIAGEN™, São Paulo, Brazil) as per the manufacturer's protocol. For reverse transcription, DNase-treated RNAs were incubated with 200 U of M-MLV (Thermo Fisher Scientific™). After the incubation of the reaction for 50 min at 37 °C, the enzyme inactivation process was performed for 15 min at 70 °C. Samples of cDNA were kept at -20 °C till they were processed.

Quantitative Reverse Transcription Polymerase Chain Reaction (RT-qPCR)

RT-qPCR was conducted using Kapa Sybr® Fast qPCR kit (Biosystems, Carlsbad, California, USA) and StepOne

ISSN: 0973-5038
DOI: https://doi.org/10.24321/0973.5038.202308

Real-Time PCR System – Applied Biosystems® (Carlsbad, California, USA) was used to perform the reaction. To adapt VHVP-RVGP quantification, first, a standard curve was obtained. After that, six dilutions were performed, producing the standard dilutions with 6×10^6, 6×10^5, 6×10^4, 6×10^3, 6×10^2, or 60 copies in every 3 µL. Next, as mentioned by Puglia et al., three duplicates of all standard dilutions were used in four separate runs.[10] Amplifications were then carried out using the following temperature profile: 95 ºC for 10 min and 40 cycles (95 ºC for 15 s, 53 ºC for 10 s, and 60 ºC for 15 s). Subsequently, melting curves were measured from 70 to 95 ºC. Finally, the absolute quantification of RNA-RVGP was determined by a mathematical model.

Transduction on Mammalian Cells

At a density of 8×10^5 cells/well, the target cells Huh-7 were planted in 6-well plates and incubated overnight at a temperature of 37 °C. The cells were treated with varying doses of viral supernatants and were allowed to incubate for three hours. After adding fresh medium, the cells were incubated for 48 hours at 37 °C.

Western Blotting Assays

Western blotting experiments were conducted to detect Gag-Pol of MLV and E1 and E2 of HCV in pseudoparticles. Centrifugation (300 g/10 min) was used to collect the co-transfected HEK 293T cells, which were then resuspended in lysis buffer (200 µL) (M-PER Insect Cell Protein Extraction Reagent, Thermo Fisher Scientific, Vilnius, Lithuania) as instructed by the manufacturer. After being extracted from the co-transfected cells' supernatant, VHVP was purified using high sucrose density cushions by the process of ultracentrifugation.

SDS-PAGE was used to separate protein samples for Western blot investigation as per the Novex® NuPAGE® SDS-PAGE Gel System, Thermo Fisher Scientific™ (running to 100 V/45 minutes). They were then transferred to a nitrocellulose membrane (GE Healthcare, Uppsala, Sweden). After blocking the membranes for an hour at room temperature with 3% non-fat dry milk in PBS-Tween 20, the membranes were incubated with primary antibodies against Gag, E1, and E2 at a 1:1000 dilution. Incubation was performed with secondary peroxidase-conjugated anti-mouse or -rabbit antibody, at room temperature for 1 hour. After this, incubation was carried out using ECL Western Blotting reagent (ECL Prime Western Blotting Detection Reagent, GE Healthcare) as suggested by the manufacturer.

VHVP-RVGP - Huh-7 Cells Interaction

After being planted at a density of 8×10^5 cells/well in 6-well plates, the Huh-7 were incubated overnight at 37 °C. The cells were transduced with 10 VHVP-RVGP/cell. The supernatant was collected every 2 hours until to complete 10 h post-transduction and 24 h post-transduction. The samples were analyzed using RT-qPCR. The over-time degradation of VHVP-RVGP was also assessed.

ELISA

The RVGP concentrations in the cell cultures were determined using an ELISA kit (Institute Pasteur, Paris), as suggested by the manufacturer. The samples, Huh-7 were harvested, centrifuged at 700 g for 5 min, and resuspended in 1 mL of lysis buffer (25 mM Tris, pH7.4, 25 mM NaCl, 5 mM $MgCl_2$) containing 0.2% Nonidet P-40 detergent (Fluka, Cat. No. 74385). The samples were mixed every 15 min during the incubation period (1 h at 4°C), followed by centrifugation for 5 min at 10,600 g for sedimentation of the cell debris. ELISA was performed (in duplicate). The samples were measured at 492 nm using a LabSystems MultiSkan MS photometer. The RVGP content was calculated using a calibrated standard curve obtained using purified RVGP (supplied in the RVGP ELISA kit).

Results

RT-qPCR Optimization

The VHVPs produced were quantified using the calibration curve. The fundamental prerequisites for RT-qPCR, including a standard curve slope ranging from -3.3 to -3.8, the usage of a minimum of five standard dilutions, an R^2 value of 0.99, and an efficiency of 92% (data not shown) were demonstrated by the standard curve.

Three duplicates of a single cDNA sample from a VHVP-RVGP pool were amplified in independent PCR runs to evaluate the reproducibility of RT-qPCR.

The estimated amounts of VHVP-RVGP produced were @ 3.6×10^4 RNA-RVGP copies/µL.

Characterization of the Virus Hybrid Vectored Particles

To characterize the VHVP, we examined the expressions of Gag-Pol polyproteins and E1 and E2 glycoprotein 48 hours post co-transfection in HEK 293T cells. The supernatant and cell lysates were analyzed using western blotting analysis (Figure 2). The E1 glycoprotein (@ 30 kDa) was confirmed in the purified VHVP, and the co-transfected lysate cells (Figure 2A) although E2 glycoprotein was only observed (@ 70 kDa) in the cell lysate (Figure 2B) indicating that the VHVP does not have the E2 glycoprotein. The mature Gag-Pol polyproteins (@ 30 kDa) were detected predominantly in the supernatant, which suggested that the Gag-Pol polyproteins were incorporated into the VHVP (Figure 2C).

ISSN: 0973-5038
DOI: https://doi.org/10.24321/0973.5038.202308

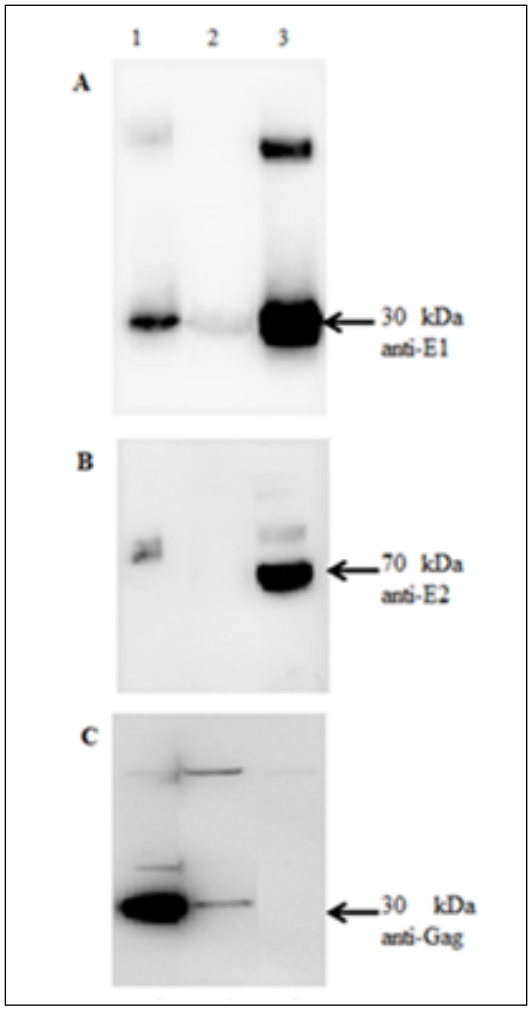

Figure 2.Characterization of the Production Process of the Virus Hybrid Vectored Particles. The proteins were collected from supernatant and cell lysate of HEK 293T cells cultured 48 hours post transfections; The Gag-Pol, E1 and E2 were detected using anti-E1 (A), anti-E2 (B) and anti-Gag (C) antibodies. Lane 1 represents the purified VHVP, Lane 2 represents the supernatant of HEK 293T transfected only pCMV-Gag-Pol and Lane 3 represents cell lysate of HEK 293T transfected only pCMV-Gag-Pol. Arrows show the molecular weight of the detected proteins

These results indicate that the VHVP has Gag-Pol and E1 proteins, but does not have E2 in its composition.

VHVP-RVGP Entry in Huh-7 Cells

To investigate the entry of VHVP into the Huh-7 cells, RNA-rabies glycoprotein was quantified in the supernatants before and after transduction. It was possible to observe that RNA titration was of ≈ 10^4 RNA-RVGP copies/µL up to 24 hours post-transduction, which suggests that only a small part, about 10% of VHVP, was able to enter the target cells after 2 hours post-transduction (Figure 3). This experiment was performed once with single samples.

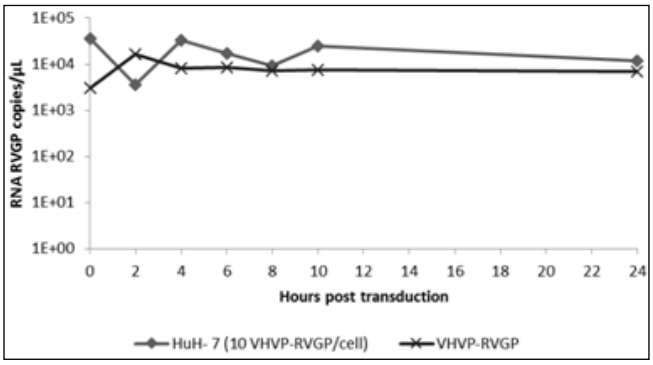

Figure 3.Quantification of RNA-RVGP Outside of Huh-7 Cells during Transduction Assay. Cells were transduced with VHVP-RVGP and RNA of VHVP's supernatant was extracted at different times for RTq-PCR evaluation of RVGP. This experiment was performed once with single samples

Expression of RVGP in Huh-7 Cells

Preliminarily, Huh-7 cells were seeded at an initial density of 8×10^5 cells/well and different concentrations of VHVP-RVGP/cells were used in the signal transduction assays. Transductions with 0.3, 2.5, 10, and 25 VHVP-RVGP/cells showed higher concentrations of RVGP expressed by Huh-7 at 48 hours post-transduction (not shown at 24 hours post-transduction). Transduction with 0.3 VHVP-RVGP/cell showed 272 ng of RVGP/10^7 cells. Transduction with 2.5, 10, and 25 VHVP-RVGP/cell showed lower expressions of RVGP (< 100 ng/10^7 cells) than 0.3 VHVP-RVGP/cell. Considering the variations intrinsic to the ELISA and that the results obtained were between 50 and 100 ng/10^7 cells, it can be inferred that there were no differences between the VHVP-RVGP/cell used. This experiment was performed three times in different periods. Figure 4 shows the average of three single samples.

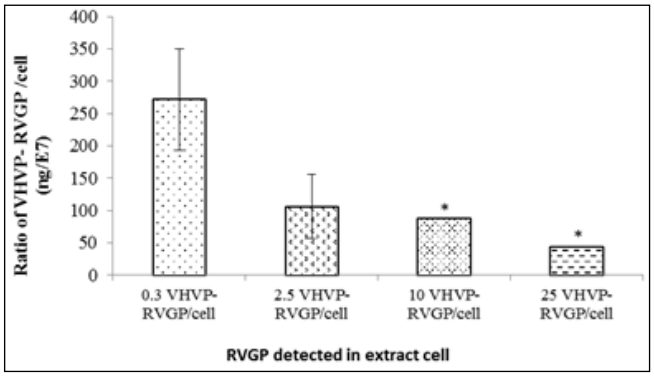

Figure 4.RVGP Expression in Huh-7 Cells Transduced with VHVP-RVGP. Different concentrations of VHVP-RVGP were used to transduce Huh-7 cells and RVGP expression was evaluated by ELISA. This experiment was performed three times in different periods. This figure shows the average of three single samples. *These experiments were performed with a single sample

ISSN: 0973-5038
DOI: https://doi.org/10.24321/0973.5038.202308

Discussion

The HCV pseudoparticles have been used to study the mechanism of hepatitis C virus infection. This system uses reporter genes such as green fluorescent protein and luciferase, which can be monitored by simple methods.[8]

This study described the use of the VHVP system for the production of virus hybrid vectored particles capable of carrying RNA-RVGP (VHVP-RVGP). The VHVP can also be used for gene transfer purposes, but their applications are limited or directed because the use of HCV glycoproteins makes this system specific to hepatic cells. Therefore, the VHVP system can be optimized and it may be useful to deliver RNA vaccines in the target cell, and this characteristic is a differential for this system in relation to virus-like particles.

Multiprotein complexes known as Virus-Like Particles (VLPs) imitate the structure and arrangement of authentic natural viruses;[11] they do not contain a viral genome, and cannot replicate.[12]

The study demonstrated that a series of standardizations were necessary to optimize the production and titration of VHVP-RVGP, as well as for the expression of RVGP. Currently, total quantification methods of HCV pseudoparticles are conducted by estimate, that is, quantification methods are performed by approximation.

Methods based on antigens are routinely used for the quantification of VLPs; in contrast, RT-qPCR cannot be used for this purpose due to the absence of genetic material.[13] The RNA-carrying system presented in this article allows the use of additional methods for titration of these VHVP by RT-qPCR.

Quantitative real-time technology has been used mainly for the quantification of mRNA targets.[14–18] The production of SFV-VRPs (non-replicating particles of Semliki Forest Virus) obtained in serum or serum-free conditions was evaluated by RT-qPCR.[10]

Our data demonstrated that titration of VHVP was standardized, and a quantification of @ 3.6×10^4 RNA-RVGP copies/µL was obtained using Vivaspin 2 to concentrate the VHVP-RVGP. The produced cotransfection batches presented similar values of RNA-RVGP copies/µL, showing that lipofection is a reproducible method to produce VHVP-RVGP.

Results showed that only a small part (about 10%) of the VHVP-RVGP produced in HEK 293T cells was able to enter into the cells (Figure 3) considering that no significant reduction was observed in the number of RNA-RVGP copies in the supernatant at different times post-transduction. The different concentrations of VHVP-RVGP/cell, used to transduce Huh-7 cells, showed similar RVGP expression levels (Figure 4). Only 0.3 VHVP-RVGP/cell increased 2.5 times in the RVGP expressions, showing that optimization strategies can be performed.

The western blot analyses showed that the VHVP were being formed by Gag-Pol of MLV and were able to incorporate into the membrane proteins as vesicular stomatitis virus glycoprotein (data not shown). However, the main protein, E2 of HCV, was retained on cell lysate, and only a small part of it was able to be incorporated into the VHVP-RVGP membrane (Figure 2).

Dubuisson et al.[19] reported the kinetics of association between El and E2 and indicated that the formation of stable E1-E2 complexes occurs very slowly and HCV glycoproteins were not found on the cell surface.[19,20] Many studies have shown that E1 cooperates with E2 in the formation of an E1-E2 function complex that is essential for hepatitis C virus entry,[21–23] which may explain the difficulty in expressing the RVGP protein in this system. In the case of viral glycoproteins, misfolded, disulfide-linked aggregates and inefficient assembly of HCV El-E2 complexes are frequent occurrences.[24]

Furthermore, the ER-localized protein disulfide-isomerase may rearrange previously generated disulfide bonds in order to produce correctly folded El and E2 glycoproteins. The time needed for half-maximal association between E1 and E2 can range from 60 to over 165 min, depending on the HCV genotype.

Considering that sustained association and specific recognition often depend on appropriately folded subunits, our results imply that the rate of HCV E1 and E2 folding, which most likely is dependent on interactions with molecular chaperones in the ER, may reduce the rate of complex formation.[21,22]

Two independently operating amino-terminal signal peptides direct the E1 and E2 glycoproteins to the endoplasmic reticulum.[25] On the contrary, the Gag-Pol capsid protein remains in the cytoplasm, which could indicate that it took the longest time for the E1 and E2 glycoproteins to migrate to the plasma membrane of the host cell.

Considering that the Gag-Pol capsid protein can be easily assembled and is able to bud the cell host, perhaps a longer time to realize the glycosylation process of E1 and E2 may be required. In addition, the time to collect VHVP in the supernatant (48 hours post-co-transfection) might not have been ideal for incorporating the E1 and E2 glycoproteins into the VHVP. Therefore, this process can be optimized to produce a VHVP-RVGP of better quality.

In addition, the VHVP system can be improved with the use of a self-replication and self-translational RNA molecule that only depends on the host cell machinery to function,[26] as the system based on the Semliki Forest Virus.

ISSN: 0973-5038
DOI: https://doi.org/10.24321/0973.5038.202308

The study data demonstrate promising results in incorporating membrane proteins and packing and delivering RNA. Improvement of this system will help in the induction of both humoral and cellular immune responses, and it can also be useful in providing targeted (cellular/ tissue/ in vivo) delivery of proteins, peptides, and nucleic acids. With the VHVP herein described, this could become an interesting system in cancer treatment and gene therapy.

Conclusion

In conclusion, this study shows the efficiency in producing VHVP from Gag-Pol of murine leukemia virus and their capacity to pack and deliver the RNA of rabies virus glycoprotein. Accordingly, the RVGP protein expression was detected at low levels because of the ineffectiveness of the E1-E2 complex and consequent ineffective transduction of the VHVP. Therefore, the optimization of this system should be performed.

Acknowledgements

The authors are grateful to Dr François-Loïc Cosset (INSERM, France) for providing the plasmids used in this work, Dr Ana Sofia Coroadinha and Dr Hugo Soares for the western blotting assays, and Ms Sandra Patiño for all her assistance during the master's course.

Source of Funding: This study was financially supported by the National Counsel of Technological and Scientific Development (CNPq) (grants nº. 152538/2012-7), Sao Paulo Research Foundation (FAPESP) (grants nº. 2009/08038-1), and Butantan Foundation. CA Pereira was a recipient of a CNPq 1A senior fellowship.

Conflict of Interest: The authors declare that they have no conflict of interest.

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ISSN: 0973-5038
DOI: https://doi.org/10.24321/0973.5038.202308