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Antiviral activity of N-ω-Chloroacetyl-L-Ornithine on in vitro replication of the Chikungunya virus.

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Abstract.

The infections caused by Chikungunya virus (CHIKV), genus Alphavirus, have become a health problem around the world, due to this virus's widespread occurrence and high morbidity rate and the absence of vaccines or antiviral drugs. In this study, we analyzed a competitive inhibitor of ornithine decarboxylase—an enzyme that is key in the biosynthesis of polyamines (PAs), N-ω-chloroacetyl-L-ornithine (NCAO), which is a possible inhibitor of CHIKV replication because intracellular polyamines participate in the in vitro transcription and translation of CHIKV. NCAO does not have any cytotoxic effect on C6/36 cells even at 1000 μM at 72 h post-exposure. However, in Vero cells, a cytotoxic effect was present above 380 μM at 48 h post-exposure, which was considered when determining the inhibitory effect on viral replication. In this work, we demonstrate that NCAO inhibits the replication of CHIKV in Vero and C6/36 cells in a dose-dependent manner, causing a decrease in the PFU/mL of at least 4 logarithms ($p < 0.01$) in both cell lines. Viral yields were restored by the addition of exogenous polyamines, mainly putrescine. The HPLC analyses showed that NCAO decreases the content of intracellular PAs, even though mainly spermidines

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and spermines are present in infected cells. NCAO inhibits CHIKV replication by depleting the intracellular polyamines in Vero and C6/36 cells, suggesting that this compound is a possible antiviral for CHIKV infections.

Keywords: Chikungunya Replication, Antiviral, N-ω-Chloroacetyl-L-Ornithine, Polyamines.

1. Introduction

Chikungunya virus (CHIKV) is a re-emergent pathogen transmitted by the bite of female mosquitoes of the genus *Aedes*. This virus belongs to the genus Alphavirus and has a positive-sense single-stranded RNA with a genome measuring around ~ 11.8 kb (Parashar et al., 2014). CHIKV infections have been a public health problem since 2005 and have spread to new areas, causing disease around the world; despite the reported cases and economic losses, however, no Chikungunya vaccines are currently included in national immunization systems, and current therapies are based on the administration of analgesics, antipyretics, and anti-inflammatory drugs to relieve symptoms (Silva et al., 2017). On the other hand, it was shown that several DNA and RNA viruses, including Chikungunya, require intracellular polyamines to carry out their replication. These are small, positively charged molecules derived from arginine that are involved in multiple cellular processes, such as cell proliferation (Pegg, 2009; Kang et al., 1994), DNA and RNA stabilization (Thomas et al., 1995; Kumar et al., 2009), cellular stress (Mitchell et al., 1998), cellular signaling (Iyer et al.,1997), and other functions in eukaryotic cells. It was found that the continuous synthesis of PAs is a requirement for some viruses, such as Vaccinia and Cytomegalovirus; when the biosynthetic pathways of polyamines are interrupted by an inhibitor such as difluoromethylornithine (DFMO), viral performance is seriously affected (Frugier et al., 1994). RNA viruses employ similar mechanisms to neutralize nucleic acids. Studies on the Semliki Forest virus showed that PAs are not present in the viral capsid, but when there is a depletion of PAs, there is a parallel marked reduction in the activity of viral RNA polymerase from the cells infected with these viruses (Tuomi et al., 1982). Thus, PAs promote RNA synthesis. Moreover, the depletion of PAs in cells infected with

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Chikungunya and Dengue viruses due to treatment with an inhibitor of ornithine decarboxylase DFMO or DENSpm (diethylnorspermine), an analog of PAs that induces the expression of SSAT, affects translation, one of the first steps in viral replication, as the depletion of PAs limits the expression of non-structural proteins, including that of viral polymerase and replication (Mounce et al., 2016a, 2016b). Recently, Mason et al. showed that polyamines facilitate coronavirus replication and the depletion of polyamines with Difluoromethylornithine (DFMO a FDA-approved), and significantly reduces coronavirus replication. Mason et al. (2020) suggested that polyamines are critical to coronavirus replication and represent a highly promising drug target in the current and any future coronavirus outbreaks. Thus, the pathway of PA biosynthesis could be a target for the development of possible antivirals against Chikungunya and other RNA viruses. Several pharmaceutical products directed toward the biosynthetic pathway of PAs, such as DFMO, have been subjected to clinical trials. Despite its ability to decrease intracellular PA levels in vitro, DFMO has toxicity at high concentrations in patients, so the search for possible antivirals against Chikungunya continues. Therefore, in this work, we studied whether N-ω-chloroacetyl-L-ornithine (NCAO) can inhibit the replication of this virus since NCAO is a competitive inhibitor of ornithine decarboxylase (ODC). Rodríguez-Paéz et al. demonstrated the inhibition properties of this compound, which cause cytotoxic and antiproliferative effects in human cancer cell lines that overexpress ODC but minimal cytotoxic effects in non-cancer control cells (Vargas-Ramírez et al., 2016). We demonstrated that N-ω-chloroacetyl-L-ornithine (NCAO) inhibits the replication of the Chikungunya virus in Vero and C6/36 cells in a dose-dependent manner by depleting the three intracellular polyamines. Subsequent studies will provide more information about the possible use of this compound or some derivatives as Chikunkunya antivirals.

2. Materials and methods

2.1 Cell culture. Vero cells were maintained in a Dulbecco's modified Eagle's medium / F-12 nutrient mixture (DMEM-F12; Sigma-Aldrich) supplemented with fetal bovine serum and penicillin–streptomycin, incubated at 37 °C and 5% CO_2. The C6 / 36 cells were maintained in a Minimal Essential Medium (MEM, Sigma-Aldrich) supplemented with fetal bovine serum and penicillin–streptomycin and incubated at 34 °C in the absence of CO_2.

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2.2 Viability tests. To study EC50, Vero and C6 / 36 cells were cultured and treated using different NCAO concentrations of 10, 100, 200, 300, 400, 500, and 1000 µM. NCAO was diluted with DMEM-F12 or MEM media without supplementation, and the cell viability was determined at 24, 48, and 72 h by a colorimetric assay using reduction of the tetrazolium salt (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide or MTT, with absorbance measured at 540 nm.

2.3 Viral replication inhibitory effect. The Vero and C6/36 cells were reseeded with a fresh medium of 2.5% fetal bovine serum overnight and then incubated for 48 h with NCAO before infection with the Chikungunya virus, with additional NCAO added at 24 h. Next, we performed viral titration on the Vero cells. For the restitution tests of polyamines, putrescine, spermidine, and spermine in the Vero or C6/36 cells (Sigma-Aldrich), the cells were diluted to 10 µM with a DMEM-F12 or MEM medium and supplemented after 48 h with the NCAO in the CHIKV-infected cells. All assays were performed in triplicate.

2.4 Viral infection and titration. The Chikungunya virus was obtained from the third passage in the C6/36 cells. The infection in Vero and C6/36 cells continued in the presence of the NCAO and during the addition of exogenous polyamines. The virus was diluted in a DMEM-F12 or MEM medium, respectively, without serum at a multiplicity of infection of 0.1, 0.25, 0.5, and 1 (MOI). Cells at 90% confluence were infected over 1 h and gently rocked periodically. Then, the cells were washed with PBS 1X at pH 7.4 and more medium was added. The supernatants were collected at 24 h post-infection for viral titration and quantification of putrescine, spermidine, and spermine by HPLC. Viral yield quantification was performed on Vero cells in 24 well plates, where150,000 cells/well were cultured. Serial 10-fold dilutions were prepared to a 10^{-7} dilution of virus for infection. The plates were incubated at 37 °C with 5% CO_2, with rocking performed every 10 min for 1 h. After the absorption period, an overlay (0.5% carboxymethylcellulose (CMC) diluted in culture medium) was added and incubated at 37 °C with 5% CO_2 until the presence of lytic plaques was observed. The cells were then fixed with 10% formaldehyde for 1h and stained with crystal violet-ethanol at 0.1% for 15 min. Then, the viral titer was determined by counting the wells present between 10 to100 lytic plates above the 10^{-3} dilution.

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2.5 Quantification of intracellular polyamines by HPLC in Chikungunya virus infection. The C6/36 and Vero cells were pre-treated at different concentrations of NCAO for 24 h and then infected with CHIKV in the presence of NCAO at 0.5 and 1 MOI. Supernatants and cell debris were collected at 24 h post-infection. For HPLC quantification, the pretreatment was performed with 50% trichloroacetic acid (Sigma-Aldrich) and 1 mM 1,8 diaminooctane (Sigma-Aldrich). Benzoylation of the polyamine standards and samples was carried out with NaOH 2 M (Sigma-Aldrich) and benzoyl chloride 50% (Sigma-Aldrich). The extraction was carried out with chloroform–HPLC (Sigma-Aldrich), and the compounds were evaporated with a stream of nitrogen. The HPLC analysis was performed using a C18 reverse phase column, Agilent 5 µm, at 250 x 4.6 mm. The mobile phase was a mixture of methanol and water (60:40), eluted with a linear gradient at a speed flow rate of 0.4 mL/min. Detection was performed at 229 nm at room temperature using the Agilent 1260 equipment (Morgan, 1998; Arisan et al., 2002).

3. Results

Several studies have identified ODC as a key enzyme in the biosynthesis of polyamines, which are necessary for the replication of several viruses, including CHIKV (Mouce et al., 2016a, 2016b). To determine if the replication of the Chikungunya virus was affected by the depletion of PAs in the presence of NCAO, a competitive inhibitor of ODC was used (Rodríguez-Páez et al., 1998; Correa-Basurto et al., 2009). To determine the possibility of an inhibitory effect, we pretreated the Vero and C6/36 cells with 100 and 500 µM NCAO for 24 h before infection with CHIKV at MOIs of 0.1, 0.25, 0.5, and 1 (Fig. 3A and 3B). At 24 h post-infection (hpi), the PFUs were determined, and a significant reduction (ANOVA * $p < 0.01$) was found in the viral titers in both concentrations in the C6/36 cells compared to the control (Fig.3B). Nevertheless, we only found a significant difference at 500 µM NCAO in the Vero cells (ANOVA *$p < 0.01$) (Fig.3A).

According to the previous results, we examined the kinetics of inhibition at MOIs of 0.5 and 1 for CHIKV in the Vero and C6/36 cells. We also analyzed the effects on the replication of the virus in the presence of NCAO after pre-treatment because it was previously reported that the enzyme presents a rapid turnover in mammalian cells, promoting the continuous synthesis of the enzyme, which could avoid the effects of

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the compound (Pegg, 2009). The NCAO treatment was performed at different concentrations 24 h before infection, and during the infection with CHIKV, titers were measured at 24 hpi. A significant reduction was found in the titers from the 200 µM concentration in the Vero cells (Fig.3C) and from a 100 µM concentration in the C6/36 cells (Fig. 3D), indicating dose-dependent inhibition as the concentration of the NCAO viral titers decreased (ANOVA * $p < 0.01$) (Fig.3C and 3D).

To verify whether the Chikungunya virus requires PAs to carry out its replication, the exogenous polyamines putrescine, spermidine, and spermine, or a mixture thereof, were added to the Vero and C6/36 cell cultures. Cells were treated with 300 µM NCAO and infected with CHIKV at 0.5 and 1.0 MOI while knowing the restitution in the viral titers when adding the exogenous PAs in both cell lines. The viral titers were similar to those obtained in the cells not treated with the compound (ANOVA * $p < 0.01$) (Fig. 4A and 4B).

To determine whether CHIKV requires PAs for replication and whether this requirement is affected by NCAO treatment, the concentration of intracellular PAs in the Vero and C6 / 36 cells infected (or not) with CHIKV was determined by HPLC (Fig.5). A decrease in the content of intracellular PAs in the presence of NCAO and during infection was observed at both MOIs for the controls in both Vero and C6/36 cells (ANOVA * $p < 0.01$) (Fig. 5A and 5B). Moreover, the content of intracellular PAs was the highest in Vero cells, and the polyamine found in the greatest quantity was putrescine, even in the presence of NCAO, which decreased significantly in each of the conditions. The content of spermidine and spermine decreased even in the presence of putrescine, the precursor PA. Interestingly, a significant decrease in spermidine content was also observed during infection with CHIKV in both cell lines (ANOVA * $p < 0.01$) (Fig. 5A and 5B).

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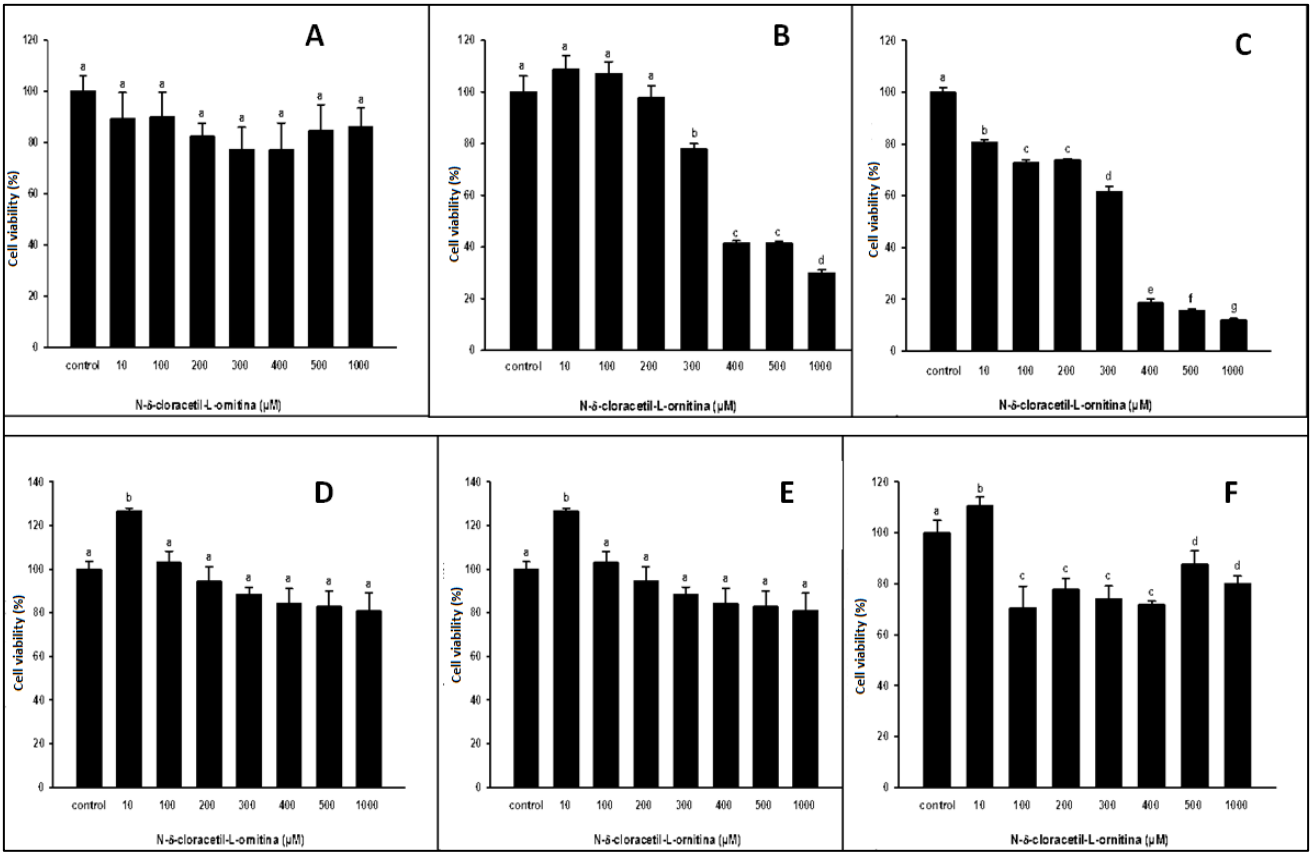

Figure 1. Viability of Vero and C6/36 cells treated with different concentrations of N-ω-chloroacetyl-L-ornithine. A: Vero Cells were treated with NCAO growths at 24 h, B: 48 h, and C: 72 h. D: C6/36 cells were treated with NCAO growths at 24 h, E: 48 h, and F: 72 h using the MTT reduction method (ANOVA P <0.05 n = 9; different letters between groups indicate significant differences).

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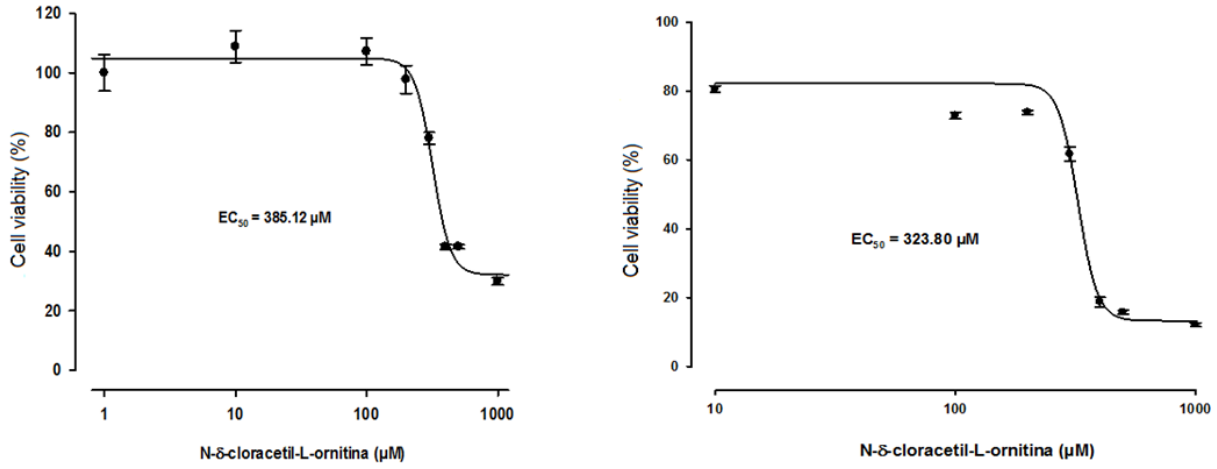

Figure 2. Concentration curve—EC_{50} response for Vero cells treated with NCAO. A: EC50 = 386.12 µM at 48 h and B: EC50 = 323.80 µM at 72 h.

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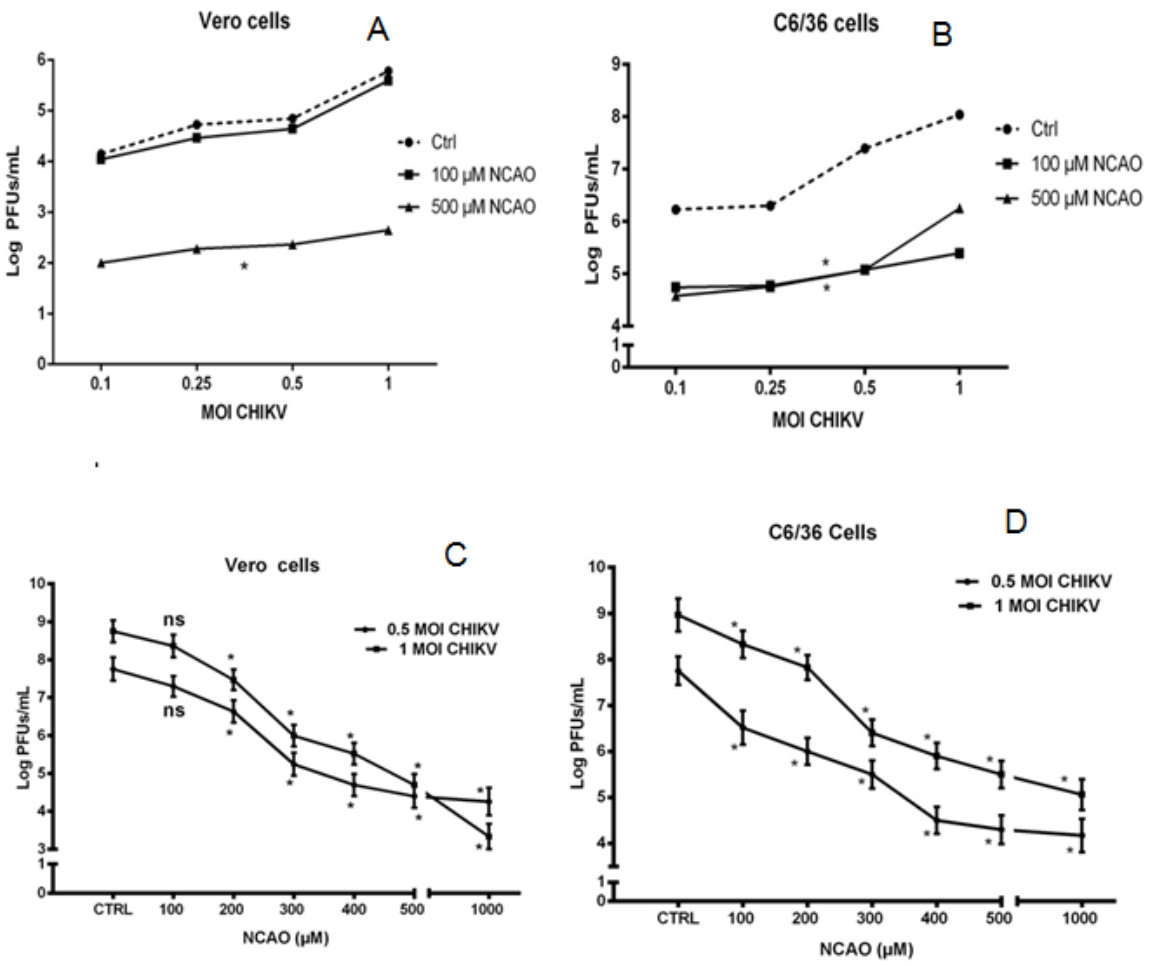

Figure 3. The NCAO affects the replication of Chikungunya virus. A and B: Vero and C6 / 36 cells were pretreated at 100 and 500 µM NCAO for 24 h and subsequently infected at 0.1, 0.25, 0.5, and 1 MOI with CHIKV. T, the titers were determined at 24 h post-infection in Vero cells. C: Vero cells and D: C6 / 36 cells pretreated at 100, 200, 300, 400, 500, and 1000 µM NCAO before and during infection with CHIKV at 0.5 and 1 MOI. Titers were determined at 24 hpi in Vero cells (ANOVA * $p < 0.01$, $n = 3$, ns = No significant difference).

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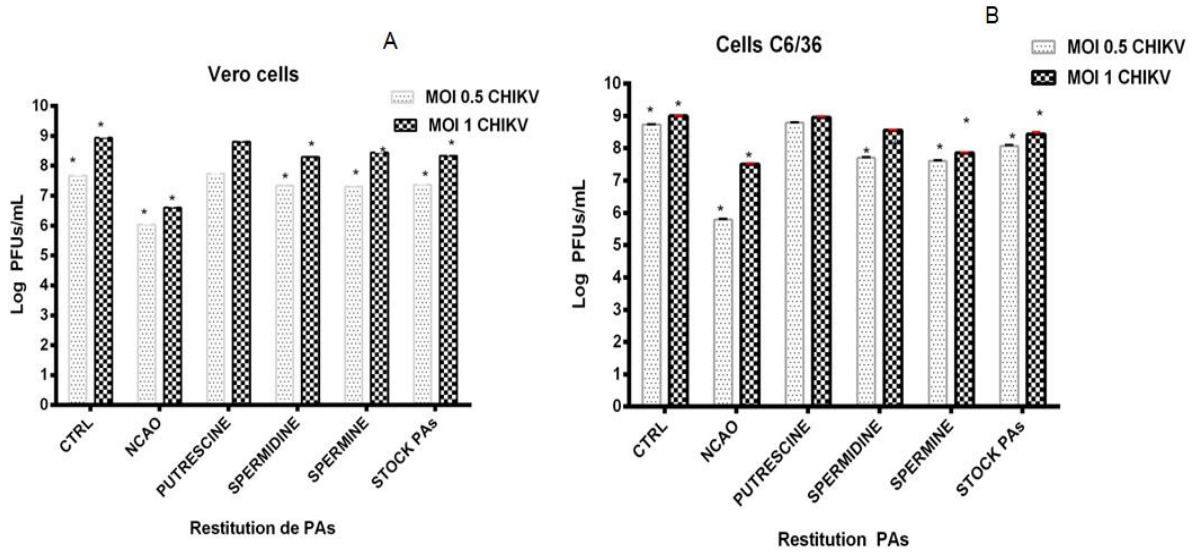

Figure 4. Restitution of CHIKV titers via the addition of PAs in Vero and C6 / 36 cells. A: Vero cells and B: C6 / 36 cells pretreated at 300 µM of NCAO and the addition of 10 µM PAs during infection with CHIKV at 0.5 and 1 MOI. Titers were determined at 24 hpi in Vero cells. A triplicate test was carried out (ANOVA * p <0.01 n = 3, ns = No significant difference).

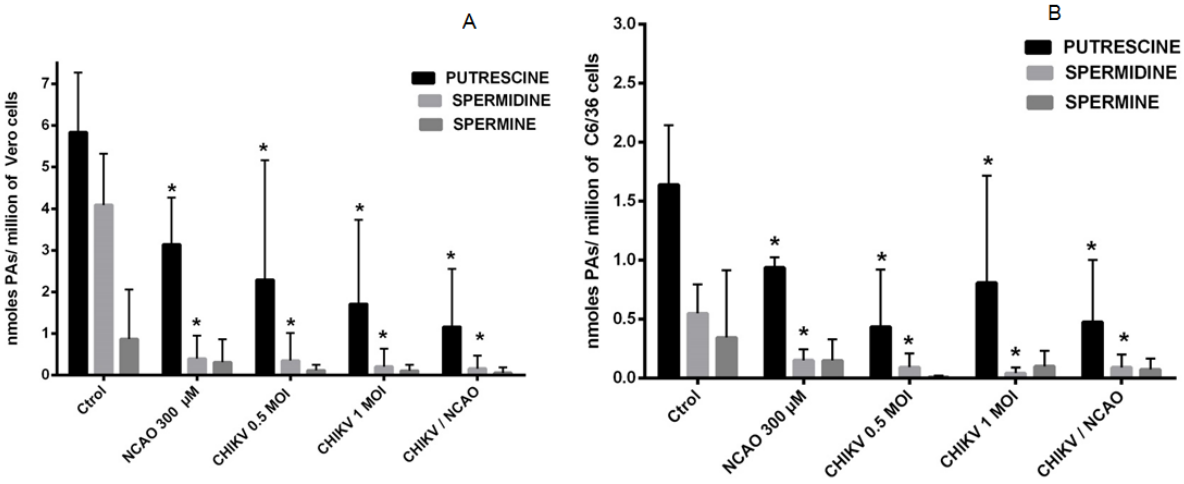

Figure 5. Content of intracellular PA in Vero and C6 / 36 cells. A: The Vero and B:C6/36 cells were previously treated with 300 µM NCAO and infected to 0.5 and 1 MOI with CHIKV. The determination of intracellular PA content was done by HPLC. The quantification of the content of the intracellular PA was performed by HPLC (ANOVA * p <0.05 n = 3, ns = No significant difference).

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4. Discussion

CHIKV infections are a public health problem due to the increase in the number of cases spread across several continents (Raiza et al., 2018). The widespread dissemination of CHIKV, its associated high morbidity rate, the lack of available treatments, and the limited knowledge about the molecular mechanisms involved in the replication process provide an opportunity for the investigation of these topics to facilitate the development of antivirals or effective vaccines for control of the infection generated by CHIKV (Silva, Dermody, 2017). Several compounds have been proposed, including chloroquine (Brighton, 1984), ribavirin (Leyssen et al., 2008), arbidol (Delogu et al., 2017), harringtonine (Kaur et al., 2013), suramin (Albulescu et al., 2015), and silymarin (Lani et al., 2015), which act in different stages of the viral replication cycle. However, these options have certain disadvantages that make them ineffective for use in CHIKV infections (Mishra et al., 2016). Mouce et al. (2016a, 2016b) showed that PAs, putrescine, spermidine, and spermine are important for the replication of a large variety of RNA viruses, including Chikungunya, and also participate in other molecular processes, such as packaging of the viral genome. In DNA viruses, polyamines stabilize the negatively charged genome within the virion particle—e.g., the bacteriophage T7 virions cytomegalovirus (CMV) (Kalejta, 2008) and vaccinia virus (Lanzer, Holowezak., 1975). Therefore, we evaluated whether N-ω-chloroacetyl-L-ornithine has an antiviral effect by inhibiting the replication of CHIKV (since it is a competitive inhibitor of ornithine decarboxylase), thereby decreasing the biosynthesis of PAs (Vargas-Ramírez et al., 2016). We demonstrated, through kinetic inhibition assays, that NCAO inhibits the replication of the Chikungunya virus in Vero and C6/36 cells in a dose-dependent manner (Fig.3A and 3B) and that this inhibition is due to a depletion of the intracellular PAs in both cell lines, as demonstrated by the PA HPLC quantification (Fig. 5). Moreover, the inhibition increased when more NCAO was added to maintain the depletion of PAs (Figure 3C and 3D). Thus, there is a relationship between the content of PAs and viral replication. All assays were performed at 24 h after NCAO exposition because NCAO did not significantly affect the cellular viability in all concentrations examined. To confirm that the inhibition of viral replication is not due to the possible cytotoxicity of NACO or a decrease in the viability of Vero cells, these cells were subjected to different concentrations of NCAO, and, after 24 h, they were washed, as was done in the cultures infected with Chikungunya virus. Then, NCAO was added at the original concentration (supplementary material). After

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another 24 h, the viability was measured, and the recovery viability was observed to be due to the nutrients in the added culture medium. In this way, we determined that the observed inhibition was due to the effect of NCAO on polyamines and not due to cytotoxicity. These results match those described by Mouce et al. (2016a, 2016b), who demonstrated that a decrease in viral replication occurs due to the depletion of polyamines in the cell lines C6/36 and BHK21 and Vero cells induced by inhibitors of PA synthesis. The suicide inhibitor of ODC, difluoro-methyl ornithine (DFMO), and diethyl-nospermine (DENSpm), an activator of SAT1, induce the exhaustion of spermidine and spermine via the induction of SAT1 (Mounce et al., 2016a, 2016b). The results also indicated that the decrease in PAs generated by these compounds exerts a negative effect in vivo and in vitro on the replication of various RNA viruses, including Chikungunya (Mounce et al., 2017a, 2017b). However, the great potential of DFMO to be used to combat viral infections is overshadowed by the adverse effects that would occur under high doses of this compound to counteract the replacement of the ODC (FL Meyskens et al., 1986). Moreover, the administration of type I interferon to induce STAT1 activity could represent a high cost. In this sense, NCAO has advantages because it is less toxic and could be considered, based on in vivo studies, as an antiviral against infection with the Chikungunya virus.

In the present work, we demonstrated that CHIKV requires polyamines for replication. When adding exogenous PAs, the CHIKV titers were restituted in both cell lines pretreated with NCAO (Fig.4). Therefore, NCAO inhibits viral replication by blocking the PA biosynthesis pathway, which is in agreement with Mouce's findings (Mouse et al., 2017a, 2017b) on the restitution of viral titers in different viruses like Chikungunya when biogenic PAs are added after pretreatment with DFMO (Mounce et al., 2016a, 2016b). To demonstrate which of the PAs would have the best effect, putrescine, spermidine, and spermine were individually added, demonstrating a better restitution of the viral titers with putrescine. This suggests that putrescine is more easily captured by the cells and used as a precursor for the synthesis of the other polyamines, which could be demonstrated by quantifying spermidine and spermine in the cells exposed to putrescine.

To rule out the possibility that viral replication was diminished by low viability, the viability of Vero or C6/36 cells in the presence of NCAO was determined. It was found that NCAO did not exert a cytotoxic effect during treatment (Fig. 1D, 1E, and 1F). However, the viability in Vero cells decreased (Fig. 1A, 1B

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and 1C) at concentrations above EC50 at 48 and 72 h (Fig.2). Interestingly, the intracellular PAs were more numerous in the Vero cells than in the C6/36 cells (Fig.5), which raises questions about the efficiency of replication, since this virus requires PAs and is replicated more efficiently in C6/36 cells. Furthermore, it was described that the enzymes involved in the synthesis and catabolism of PAs are conserved throughout the various kingdoms. Recent studies of polyamines in the mosquito Aedes aegypti showed that the content of PAs likely depends on the enzyme aaNAT5b, which is an acetyltransferase (SAT). SAT could act as a viral restriction factor by decreasing intracellular PAs (Guan et al., 2018). However, there are likely to be other mechanisms in the cells that allow the virus to replicate successfully in the absence of polyamines.

Regarding the concentration of PAs in Vero and C6/36 cells during infection with CHIKV (Fig. 5), a decrease in the presence of NCAO was observed at the two MOIs tested (Fig. 5A and 5B), which was expected since the NCAO decreased the pools of intracellular Pas by inhibiting the ODC (Vargas-Ramirez et al., 2016). Moreover, spermidine and spermine were diminished because the virus consumed these molecules during replication. In several experimental studies, it was demonstrated that the activity of the RNA-dependent RNA polymerases (RdRP) of the Chikungunya (Mounce, et al., 2017a) and Semliki Forest viruses (SFV) (Tuomi et al., 1982) are diminished in the absence of polyamines and that their activity is restored by adding spermidine (Mounce, et al.,2017a, 2017b). Likewise, it is known that spermidine is important in one of the most critical steps of viral replication—translation—since the depletion of PAs limits the synthesis of non-structural proteins including viral polymerase (Mounce et al., 2016a, 2016b), as spermidine plays an important role specifically in the initiation and lengthening of translation through hypo functioning the 5A initiation factor (eIF5A) (Nishimura et al., 2005). Indeed, bunyaviruses and alphaviruses replicate under supplementation of 100 nM polyamines in polyamine-depleted cells. Thus, these viruses may have evolved mechanisms to take advantage of polyamines, even at low concentrations (Firpo et al., 2020).

In this work, it was observed that putrescine is found in great quantity, even in the presence of NCAO, which suggests that this cell uses other mechanisms to assure the content of this PA. Currently, the mechanism by which PA transport is carried out has not been fully elucidated. However, it is known that

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the transport of putrescine to the interior of the cell could occur through the exporter SLC3A2, which is present in the cell membrane (Nowotarski et al., 2013). Interestingly, in the treatment with NCAO, the spermidine and spermine content decreased even in the presence of putrescine—the precursor PA. This result suggests that NCAO could interact with some other enzymes of the biosynthetic route, in addition to ODC. N-ω-chloroacetyl-L-ornithine (NCAO) inhibited Chikungunya virus replication in a dose-dependent manner in Vero and C6/36 cells by depleting the intracellular polyamines. Spermidine may have a more complex role in the process of translation and transcription of the virus. Firpo et al. suggest that polyamines are critical to coronavirus replication and represent a highly promising drug target for the current, and any future, coronavirus outbreaks (Firpo, 2020). Subsequent studies should provide more information about the possible uses of this compound, or its related derivatives, as antivirals for RNA viruses.

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Conflicts of interest/declarations of interest: none.

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Ethical Approval: The project was reviewed and approved by the biosafety committee of the Higher School of Medicine, NIP.

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