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Explorations on the antiviral potential of zinc and magnesium salts against chikungunya virus: implications for therapeutics

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Background: Chikungunya virus (CHIKV), which causes chikungunya fever, is an arbovirus of public health concern with no approved antiviral therapies. A significant proportion of patients develop chronic arthritis after an infection. Zinc and magnesium salts help the immune system respond effectively against viral infections. This study explored the antiviral potential of zinc sulphate, zinc acetate, and magnesium sulphate against CHIKV infection.

Methods: The highest non-toxic concentration of the salts (100 μM) was used to assess the prophylactic, virucidal, and therapeutic anti-CHIKV activities. Dose-dependent antiviral effects were investigated to find out the 50% inhibitory concentration of the salts. Entry bypass assay was conducted to find out whether the salts affect virus entry or post entry stages. Virus output in all these experiments was estimated using a focus-forming unit assay, real-time RT-PCR, and immunofluorescence assay.

Results: Different time- and temperature-dependent assays revealed the therapeutic antiviral activity of zinc and magnesium salts against CHIKV. A minimum exposure of 4 hours and treatment initiation within 1 to 2 hours of infection are required for inhibition of CHIKV. Entry assays revealed that zinc salt affected virus-entry. Entry bypass assays suggested that both salts affected post-entry stages of CHIKV. In infected C57BL6 mice orally fed with zinc and magnesium salts, a reduction in viral RNA copy number was observed.

Conclusion: The study results suggest zinc salts exert anti-CHIKV activity at entry and post entry stages of the virus life cycle, while magnesium salt affect CHIKV at post entry stages. Overall, the study highlights the significant antiviral potential of zinc sulphate, zinc acetate, and magnesium sulphate against CHIKV, which can be exploited in designing potential therapeutic strategies for early treatment of chikungunya patients, thereby reducing the virus-associated persistent arthritis.

KEYWORDS

zinc, magnesium, chikungunya virus, antiviral agents, therapeutics

1 Introduction

Chikungunya virus (CHIKV), an arthritogenic virus belonging to the family Togaviridae and genus Alpha virus, causes chikungunya fever (CHIKF). Infected patients recover within a week; however, chronic arthritis in some might persist for weeks to a few months. Currently, there are no antivirals approved for treating CHIKF, and the treatment focuses on supportive care. Supportive care measures include the administration of pain relievers (like acetaminophen), anti-inflammatory drugs (avoiding nonsteroidal drugs due to the potential risk of bleeding), and maintaining adequate hydration (da Cunha and Trinta, 2017). Viral persistence has been shown to be associated with arthritis (Zhang et al., 2022), and reducing the viral load might contribute to preventing the development of chronic arthritis. Currently, several plant-based extracts as well as FDA-approved drugs are being explored for their efficacy in suppressing CHIKV infections (Patil et al., 2021; Alagarasu et al., 2022; Kasabe et al., 2023). Though the recent approval of Ixchiq, the first chikungunya vaccine, by the U.S. Food and Drug Administration represents a significant advancement in preventive measures (https://www.fda.gov/, assessed on December 9, 2023), it is imperative to acknowledge that the development of new medicines remains crucial. Despite the availability of vaccines, the ongoing need for effective antiviral treatments and therapeutics underscores the importance of a multifaceted approach to combating viral infections.

Studies have reported that zinc salts, an important micronutrient, exert *in-vitro* antiviral activity against different viruses. Zinc interferes with human papillomavirus replication by inhibiting the synthesis of crucial viral proteins (Read et al., 2019). Zinc chloride inhibits the production of infectious virus particles in chicken embryo fibroblast cells infected with Sindbis virus (Bracha and Schlesinger, 1976). Zinc ions also inhibit Semiliki Forest virus (SFV) liposome fusion and particularly inhibit the formation of the post fusion E1 trimer in wild-type SFV (Corver et al., 1997; Liu and Kielian, 2012). Studies have examined the role of zinc supplements in reducing the severity and duration of viral infections, especially for the common cold and certain coronaviruses (Marreiro et al., 2022). Oral zinc supplementation has been reported to enhance the recovery rate and reduce the incidence of severity as an adjunctive treatment for rotavirus enteritis in infants (Telmesani, 2010; Jiang et al., 2016). Chelation of zinc abrogates dengue virus replication (Kar et al., 2019). Zinc ions can penetrate cell membranes and accumulate within infected cells. Within the cell, zinc exerts its antiviral effects by disrupting viral replication mechanisms and impeding the formation and release of new virus particles (Xia et al., 2021). Holzmann et al. (1988) explored the clinical efficacy of combinations of heparin and $ZnSO_4$ against the herpes simplex virus (HSV). Despite its clinical effectiveness, the underlying mechanism of the alleged antiherpetic activity remained unclear. Studies investigating the inhibitory effects of zinc salts on HSV replication indicated that zinc ions inhibited HSV DNA polymerase at a lower concentration compared to cellular DNA polymerases α and β in cell homogenates (Fridlender et al., 1978). Additionally, zinc also acts as an antioxidant, protecting cells from oxidative damage and maintaining cellular integrity and function, potentially reducing the impact of viruses on host cells (Cuajungco et al., 2021).

Magnesium is an essential mineral with potential antiviral properties. Evidence suggests that magnesium can modulate immune responses and influence viral replication, although it is not typically considered a direct antiviral agent (Mizutani et al., 1994). By aiding immune cell activation, cytokine production, and antibody response, magnesium supports a well-balanced immune system crucial for effective defense against viral infections (Tam et al., 2003).

The potential impact of supplementing zinc and magnesium, both *in vitro* and *in vivo*, on CHIKV has not been studied. In the present study, the antiviral potential of zinc sulphate, zinc acetate, and magnesium sulphate against CHIKV replication was explored using a range of experimental approaches.

2 Materials and methods

2.1 Ethics statement

Animal experiments were performed within a biosafety level-2 animal facility at the ICMR-National Institute of Virology, Pune. The research protocol was approved by both the Institutional Animal Ethics Committee (IAEC) and the Institutional Biosafety Committee. The housing and care of animals adhered strictly to the guidelines established by the Committee for Control and Supervision of Experiments on Animals (CCSEA) (IAEC number CHK 1501, approved on December 20, 2017).

2.2 Virus and cell line used

The CHIKV strain (061573, Andhra Pradesh, India), was used in all the experiments. Vero CCL-81 cells (ATCC® CCL81™) were grown in minimal essential medium (MEM) (Himedia®, India) containing 10% fetal bovine serum (FBS) (Gibco™, USA) and antimycotic antibiotic solution (Sigma Aldrich®, USA) at 37°C with 5% CO_2. Zinc sulphate (Product No. Z4750, purity ≥99.0% based on titration with EDTA), zinc acetate (Product No. 379786, purity ≥99.999% based on trace metal analysis), and magnesium sulphate (Product No. M2643, purity > 98% based on titration with EDTA) were procured from Sigma-Aldrich Chemicals for use in the study. The compounds were dissolved in double-distilled water and diluted in the culture medium before each assay.

2.3 Cell viability assessment

The cytotoxic effects of the zinc and magnesium salts were assessed as described previously (Patil et al., 2021). Briefly, Vero CCL-81 cells (3.5×10^4 cells per well) cultured in 96-well micro-titre plates were treated with a range of concentrations (450 µM to 2.5 µM) of zinc and magnesium salts. Cell viability was investigated using the 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyll tertrazolium bromide (MTT) reagent (Sigma-Aldrich™, USA) after 24, and 48 hours. CC50, the concentration of the compound needed to reduce the viability of cells by 50%, was computed using non-linear regression analysis.

2.4 Screening of anti-CHIKV activity of zinc and magnesium salts

To assess the antiviral potential of zinc and magnesium salts, the highest concentrations of the salts that did not exhibit toxicity were chosen. This assessment was conducted under three distinct scenarios: prior to infection (pre-treatment), during infection (co-treatment), and after infection (post-treatment). In each case, 2×10^5 cells *per* well were seeded in a 24-well plate and infected with a multiplicity of infection (MOI) of 0.01. After infection, free virus particles were removed by washing twice with 1x sterile phosphate-buffered saline (PBS), and the plates were then incubated for 24 hours in presence of salts or only MEM with FBS.

In the pre-treatment scenario, cells were exposed to the salts for 24 hours before infection. In the therapeutic (post-treatment) approach, cells were first infected with CHIKV for an hour and then subjected to 24 hours of drug treatment. In the co-treatment approach, the virus was incubated with salts for an hour, and the cells were infected with the virus-drug mixture. After one hour, the mixture was removed, the cells were washed with sterile PBS, and incubated for 24 hours in MEM with 2% FBS. For all conditions, virus control (VC) wells were maintained in which the infected cells did not receive any treatment. After incubation, virus quantification was performed by assessing the viral load in the culture medium using the Foci Forming Unit (FFU) assay as per previously reported protocols and conditions (Alagarasu et al., 2022; Kasabe et al., 2023).

2.5 Dose dependent anti-CHIKV activity of zinc and magnesium salts

The dose-dependent effects of zinc and magnesium salts were evaluated under post-treatment conditions. Vero cells were infected with a 0.01 MOI of CHIKV at 37°C for 1 hour. After infection, cells were washed twice with 1x sterile PBS, followed by treatment with different concentrations of salts (ranging from 10 to 100 µM) dissolved in MEM with 2% FBS. The plates were then incubated for 24 hours, with a virus control maintained without salts. After incubation, the plates were freeze-thawed, and the clarified culture supernatant was assessed for infectious virus titre using the FFU assay and for viral RNA using the RT-qPCR method as per previously reported protocols and conditions (Alagarasu et al., 2022; Kasabe et al., 2023).

The percentage reduction in FFU titre compared to the virus control was calculated for each concentration, and the 50% inhibitory concentration (IC50) was determined using non-linear regression analysis with GraphPad Prism software version 9.

2.6 Effect of zinc and magnesium salts on the percent infectivity in Vero CCL81 cells

To assess the effect of zinc and magnesium salts on CHIKV infectivity, an immunofluorescence assay (IFA) was conducted. Vero cells were seeded onto sterile cell culture coverslips in a 24-well plate at a density of 2×10^5 cells per well. The confluent monolayer of Vero cells was infected with a 0.01 MOI of CHIKV at 37°C for 1 hour. Following infection, cells were washed twice with 1x sterile PBS, after which different concentrations of salts dissolved in MEM with 2% FBS were added, while virus control was maintained without salts. The infected cells were visualized by staining with an in-house prepared anti-CHIKV monoclonal antibody (ClVE4/D9 clone) following incubation with a secondary antibody (anti-mouse IgG conjugated with FITC). The detailed assay was performed according to previously reported protocols and conditions (Alagarasu et al., 2022; Kasabe et al., 2023). Infected cells were counted, and percent infected cells were calculated for each concentration of salts with reference to the VC.

2.7 Effect of zinc and magnesium salts on the percent cell viability in CHIKV infected cells

The antiviral activity of the salts was further confirmed by investigating whether treatment with zinc and magnesium salts was able to prevent virus-induced cytopathic effects using the MTT assay. Infected cells treated with and without zinc and magnesium salts (2.5, 5, 10, 20, 40, 60, 80, and 100 µM concentrations) were subjected to the MTT assay, and percent cell viability was calculated with reference to uninfected and untreated cells.

2.8 Viral attachment and entry assay

Virus attachment and entry assays were performed to investigate the effect of zinc and magnesium salts on viral attachment and entry. In the attachment assay, salts were mixed with 1 MOI of CHIKV and incubated at 37°C for 1 hour, and Vero CCL-81 cells were infected with this virus-salt mix following 1 hour of incubation at 4°C. After 1 hour, cells were washed twice with 1x PBS and then incubated in 300 µl of MEM supplemented with 2% FBS at 37°C for 8 hours.

In the entry assay, Vero CCL-81 cells were first infected with CHIKV and incubated at 4°C for 1 hour, then washed twice with 1x sterile PBS. Zinc and magnesium salts were added to the cells, followed by incubation at 37°C with 5% CO2 for 2 hours. Afterward, cells were washed twice with 1x sterile PBS and incubated in 300 µl MEM supplemented with 2% FBS at 37°C for 8 hours.

A virus control without any salt was maintained for both the attachment and entry assays. Following incubation, the freeze-thawed clarified culture filtrates were used to determine the virus titre using an FFU assay. The FFU titres between salt-treated infected cultures and the virus control were compared to assess the effect of zinc and magnesium salts on virus attachment and entry.

2.9 Effect of time of exposure to zinc and magnesium salts and time of treatment initiation on CHIKV infection and replication

To ascertain the duration of exposure at which the salts are effective in inhibiting CHIKV replication, time of addition (TOA) and time of removal (TOR) assays were performed. Vero CCL-81

cells were cultured in 24-well plates and incubated at 37°C under 5% CO2 to facilitate adherence and growth. Cells were exposed to a 1 MOI of CHIKV for one hour at 4°C to synchronize infection and one hour at 37°C. The highest non-toxic concentrations of zinc and magnesium salts were added at 1, 2, 3, 4, and 6 hours for TOA. For the TOR assay, the highest non-toxic concentrations of zinc and magnesium salts were added immediately after virus entry and removed at 1, 2, 3, 4, and 6 hours. The cultures were then incubated in 300 µl MEM supplemented with 2% FBS at 37°C for an additional 8 hours in both cases. Positive controls with only virus-infected cells (no salts) and negative controls with uninfected cells were included for each time point with respect to TOA or TOR. After incubation, cells were freeze-thawed, and clarified culture supernatant was assessed for virus titre using an FFU assay.

2.10 Viral entry bypass assay

To find out whether the salts affect post-entry stages of the virus life cycle, an entry bypass assay was conducted in which the infection step was replaced by transfection of the cells with viral RNA. Vero CCL-81 cells (2×10^5 cells per well) were added in 24-well plates and incubated overnight in 2% FBS MEM medium at 37°C. CHIKV RNA was extracted using the RNeasy kit as per manufacturer guidelines (QIAGEN), and the transfection complex was prepared in a sterile microcentrifuge tube by combining 40 µl (≥ 2 MOI of virus titre equals to 23 ng of viral RNA) of CHIKV RNA solution with 10 µl of Lipofectamine® 2000 (Catalog No. 11668-019). The solution was mixed gently by pipetting up and down and incubated for 10 minutes at room temperature to allow the lipofectamine to complex with RNA. Opti-MEM media (50 µl) was added to the RNA tube and incubated for 30 min to form the lipofectamine-RNA complex. The growth media from the cells was carefully removed, followed by the dropwise addition of 100 µl of transfection complex to the cells and incubation at 37°C for one hour. Transfection media was removed, and salts were added and incubated for 24 hours. The freeze-thawed and clarified culture supernatants were analyzed for the expression of the CHIKV genomic RNA and negative strand RNA, which is the replication intermediate (Kaur et al., 2013). In the negative strand PCR, only a forward primer was used for reverse transcription, and for subsequent real-time PCR, both primers were used as described earlier. A FFU assay was performed to measure the titre of virus particles.

2.11 Effect of metallic salts on viral replication in mice

A total of 12 female C57BL/6 mice aged 3-4 weeks, obtained from our institutional animal breeding facility, were used in the study. The animals were categorized into four groups: virus control, $ZnSO_4$, ZnAc, and $MgSO_4$ treatment groups, each comprising three individuals (n = 3). The experiment was conducted twice for consistency in the results. These mice were chosen randomly, marked for individual identification, and housed in their cages for a minimum of one week before dosing. This acclimatization period allowed them to adapt to the laboratory conditions. To evaluate the potential anti-CHIKV effects of zinc and magnesium salts, the mice were infected with 1×10^7 FFU/ml of CHIKV through the intramuscular route. Zinc and magnesium salts were dissolved in the Milli-Q water and given orally. A dose of 30 mg/kg was given on the alternative days for 7 days, as described earlier (Davis et al., 2022). Subsequently, viral RNA copies were quantified in serum on the second, third, fifth, and seventh day post-infection (dpi). The animals were humanely euthanized, followed by cervical dislocation at the end of the study.

2.12 Molecular docking of metallic salts to CHIKV proteins

Molecular docking studies were performed on the metal ion-binding server (MIB2) to find out the interactions of zinc and magnesium ions with CHIKV proteins (http://bioinfo.cmu.edu.tw/MIB2/) (Lin et al., 2016; Lu et al., 2022). Crystal structures of mature CHIKV envelope protein (3N42) (Voss et al., 2010), nsp2 protease (4ZTB) (Narwal et al., 2018), nsp2 helicase (6JIM) (Law et al., 2019), nsp4 RdRp domain (7F0S) (Tan et al., 2022), and nsp3 ADP ribose macrodomain (3GPO) (Malet et al., 2009) were obtained from the RCSB protein data bank in PDB format and uploaded to the MIB2 online server. The proteins showing the highest binding scores with the Zn and Mg salts were analyzed for interacting residues in the active site, and their interactions with the ions were visualized using Biovia Discovery Studio version 21.1.

2.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism 9. Data were pooled from three independent experiments in triplicates (n=3/treatment groups per experiment) for analysis and reported as the mean ± SE. A non-linear regression analysis was performed to calculate the CC50 and IC50 values. The mean virus titre was represented as FFU/ml, RNA copy number, and percent infected cells and was compared using a one-way ANOVA. The results were considered statistically significant when the P values were less than 0.05.

3 Results

3.1 Cytotoxic effects of zinc and magnesium salts in Vero CCL-81 cells

The cytotoxicity of zinc and magnesium salts to Vero CCL-81 at concentrations ranging from 2.5 to 400 µM in Vero-CCL81 cells was assessed at 24-, and 48-hour time points using the MTT assay. Zinc sulphate and zinc acetate at concentrations up to 100 µM showed no toxicity, while concentrations higher than 100 µM exerted more than 20% cytotoxicity. Magnesium sulphate exhibited no observable toxicity at all the time points studied. The CC50 values in µM corresponding to various salts are shown

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in Supplementary Figure 1. The concentration of the salts that facilitated cell viability of ≥80% was subsequently selected for investigating their antiviral properties.

3.2 Zinc and magnesium salts exert anti-CHIKV activity under post-treatment conditions

The prophylactic (pre-treatment), virucidal (co-treatment), and therapeutic (post-treatment) activities of the salts were assessed at concentrations of 100 μM. The metallic salts had no effect on virus titre under pre-treatment and co-treatment conditions (Figures 1A, B). Under post-treatment conditions, a notable reduction (≥1 log10 reduction) in CHIKV titre was noticed in cultures that received the salts compared to the virus control (infected cells without any treatment). In cultures treated with zinc sulphate a reduction in virus titre from 9 ± 0.5 to 5 ± 0.5 mean log_{10} FFU/ml was observed. In cultures treated with zinc acetate, the virus titre was reduced to 4 ± 0.5 mean log_{10}FFU/ml in treated cultures from 9 ± 0.5 mean log_{10}FFU/ml in VC. In cultures that received magnesium sulphate, a four-log decrease in titre was observed (9 ± 0.5 to 5 ± 0.5 mean Log_{10}FFU/ml) (Figure 1C).

3.3 Anti-CHIKV activity of zinc and magnesium salts in a dose-dependent manner

Since a reduction in virus titre was observed under post-treatment conditions, the dose-dependent antiviral activity of zinc and magnesium salts was investigated, and the virus titre was assessed. The results revealed a concentration-dependent decrease in virus titre in terms of FFU/ml, RNA copy number/ml, and percentage of infected cells, in cultures treated with the salts compared to the VC. Based on FFU titre, the percentage virus reduction, and the IC50 values, which represent the concentration required to inhibit 50% of the virus titre, were calculated. The IC50 values for zinc sulphate, zinc acetate, and magnesium sulphate were 23.72 μM, 37.12 μM, and 27.67 μM, with selectivity index (SI) of 11.10, 6.7, and 76.72, respectively (Figures 2A–C).

3.4 Zinc and magnesium salts reduce virus infectivity

To validate the antiviral effect of zinc and magnesium salts, IFA was performed to quantify the percent infection and the capsid protein

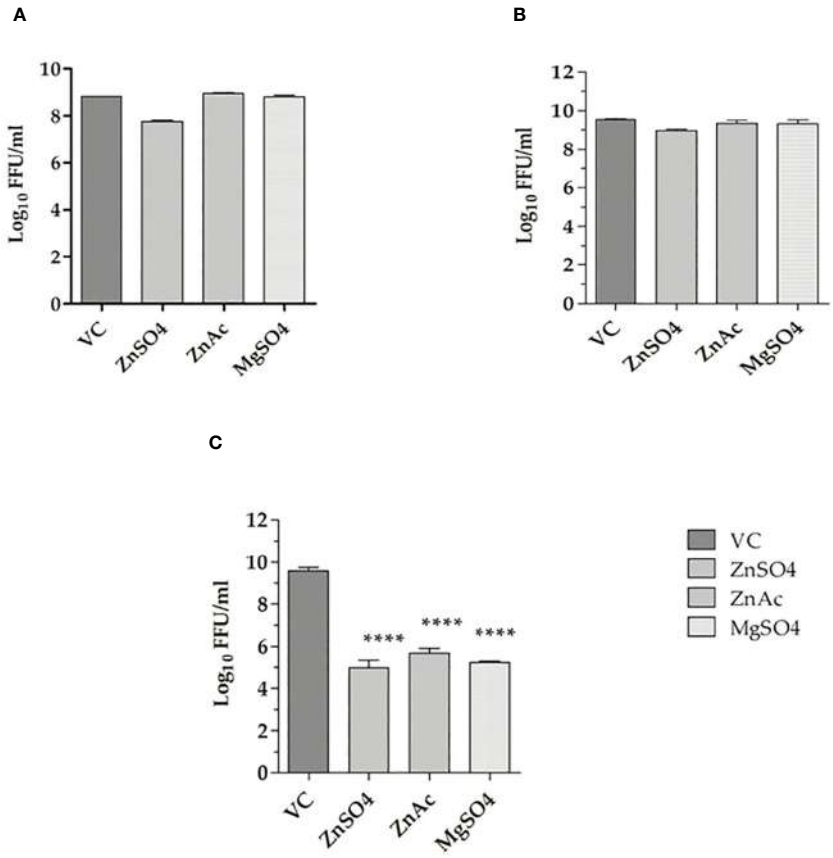

FIGURE 1
Effect of metallic salts on CHIKV replication under various treatment approaches. **(A)** Pre-treatment, **(B)** Co-treatment, and **(C)** Post-treatment. The experiments were performed in triplicates in two independent trials, and the virus titres are expressed as mean log_{10} FFU/ml ± SE. The virus output in cultures treated with zinc and magnesium salts were compared with the virus control (VC). ****p < 0.0001.

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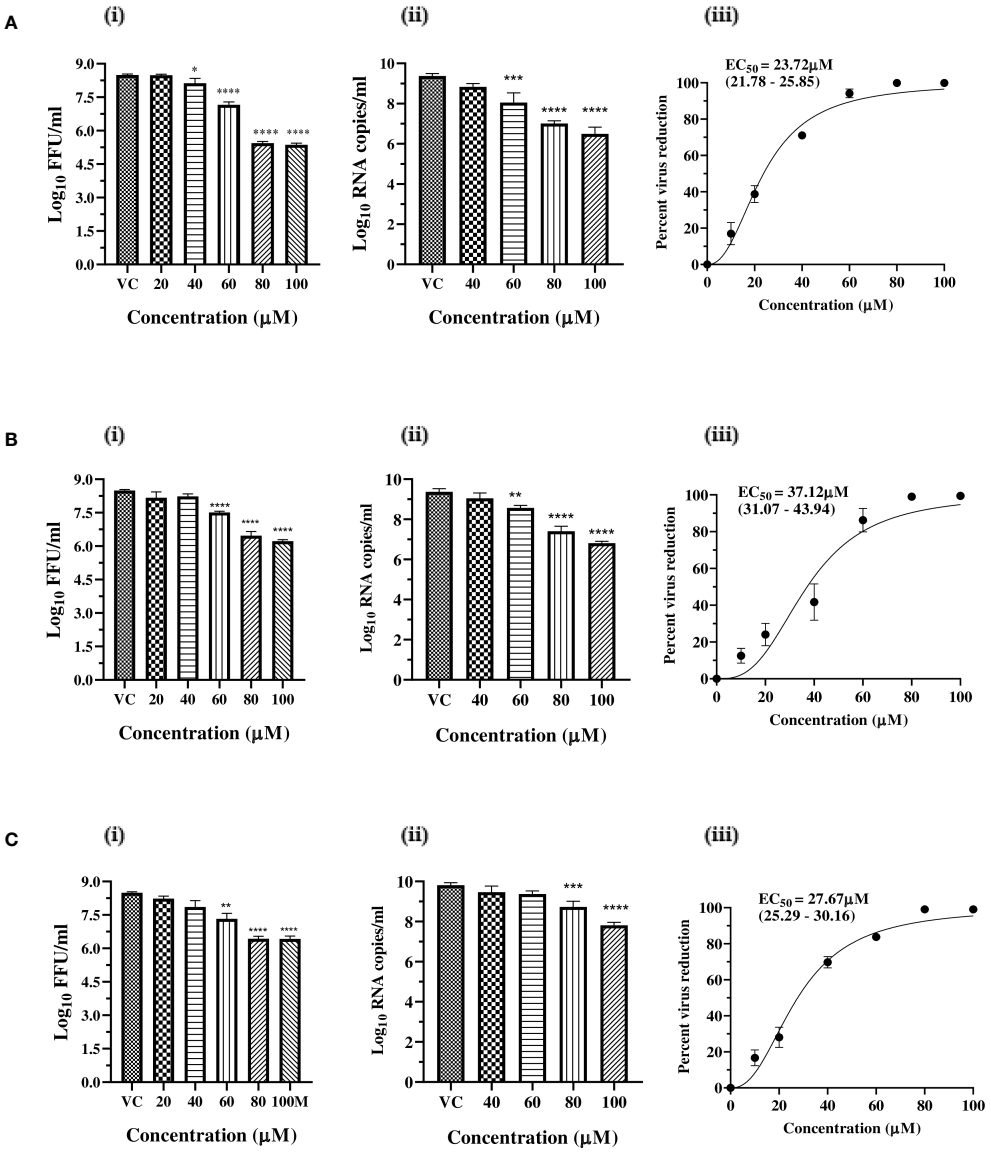

FIGURE 2
Dose dependent antiviral effects of **(A)** zinc sulphate, **(B)** zinc acetate, and **(C)** magnesium sulphate on CHIKV infection and replication. (i) Effect of treatment of Vero CCL-81 cells with zinc and magnesium salts on the production of infectious virus titre expressed as mean log_{10} FFU/ml ± SE (ii) Effect of treatment of Vero CCL-81 cells with zinc and magnesium salts on the synthesis of genomic RNA expressed as mean log_{10} viral RNA copy number/ml ± SE. (iii) Mean percent virus reduction ± SE based on FFU titre exerted by different concentrations and IC50 (95% CI). ** p<0.01; *** p<0.001; **** p<0.0001.

levels of CHIKV in infected and treated cells. A dose-dependent decrease in viral protein expression was observed in infected cultures that received zinc and magnesium treatment (Figures 3A, B).

3.5 Zinc and magnesium salts rescue cell viability in CHIKV infected cells

To find out whether treatment with metallic salts protects against virus-induced cytopathic effects, the cell viability in infected cell cultures treated with and without zinc sulphate, zinc acetate, and magnesium sulphate relative to the uninfected and untreated cells was assessed. It was observed that higher non-toxic concentrations of all the metallic salts rescued 100% cell viability in infected cell cultures compared to VC (Supplementary Figures S2).

3.6 Zinc salt inhibits CHIKV entry

To investigate the potential effects of zinc and magnesium salts during virus attachment, entry, and uncoating, entry and attachment assays were conducted. Virus attachment was facilitated by incubating cells with virus and salt mixtures at 4°C following a one-hour incubation at 37°C. A significant reduction (≥1 log10 reduction) in CHIKV titre was observed in cultures treated with the salts compared to the virus control, suggesting that

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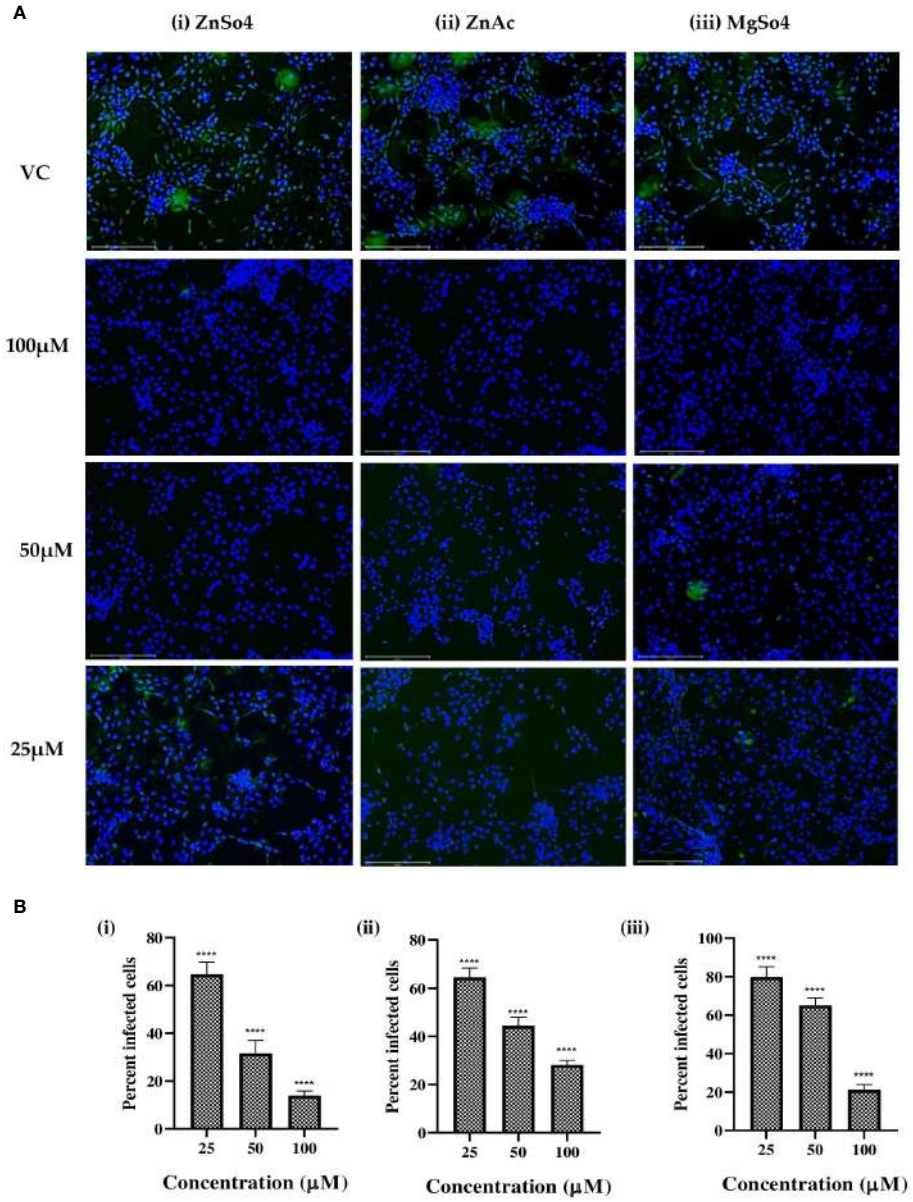

FIGURE 3
(A) Immunofluorescence images of CHIKV-infected Vero cells treated with different concentrations of (i) zinc sulphate, (ii) zinc acetate, and (iii) magnesium sulphate. Infected cells appear as green due to expression of capsid protein which is probed with a FITC labelled monoclonal antibody against the capsid protein, while uninfected cells appear as blue due to nuclear staining with DAPI. **(B)** Effect of (i) zinc sulphate, (ii) zinc acetate, and (iii) magnesium sulphate treatment on percent cell infection in Vero CCL-81 cells. **** p<0.0001.

zinc salts inhibited virus entry. However, both the salts were not effective during virus attachment (Figures 4A, B).

3.7 Zinc and magnesium salts inhibit CHIKV replication

TOA and TOR assays were conducted to investigate the impact of exposure duration and timing on the inhibitory effects of zinc and magnesium salts on CHIKV infection and replication in Vero CCL-81 cells. The experiments revealed that zinc treatment within 4 hours after virus entry, with a minimum exposure of 1 hour, significantly reduced virus titers compared to the control (VC). whereas magnesium salt treatment required a minimum exposure of 3 hours and administration within 4 hours after virus entry to effectively inhibit CHIKV in cultures treated with magnesium salts compared to the control (VC) (Figures 4C, D).

3.8 Zinc and magnesium salts inhibit CHIKV during post-entry stages

To find out whether the zinc and magnesium salts act at the level of entry and uncoating or at post-entry stages, a entry bypass

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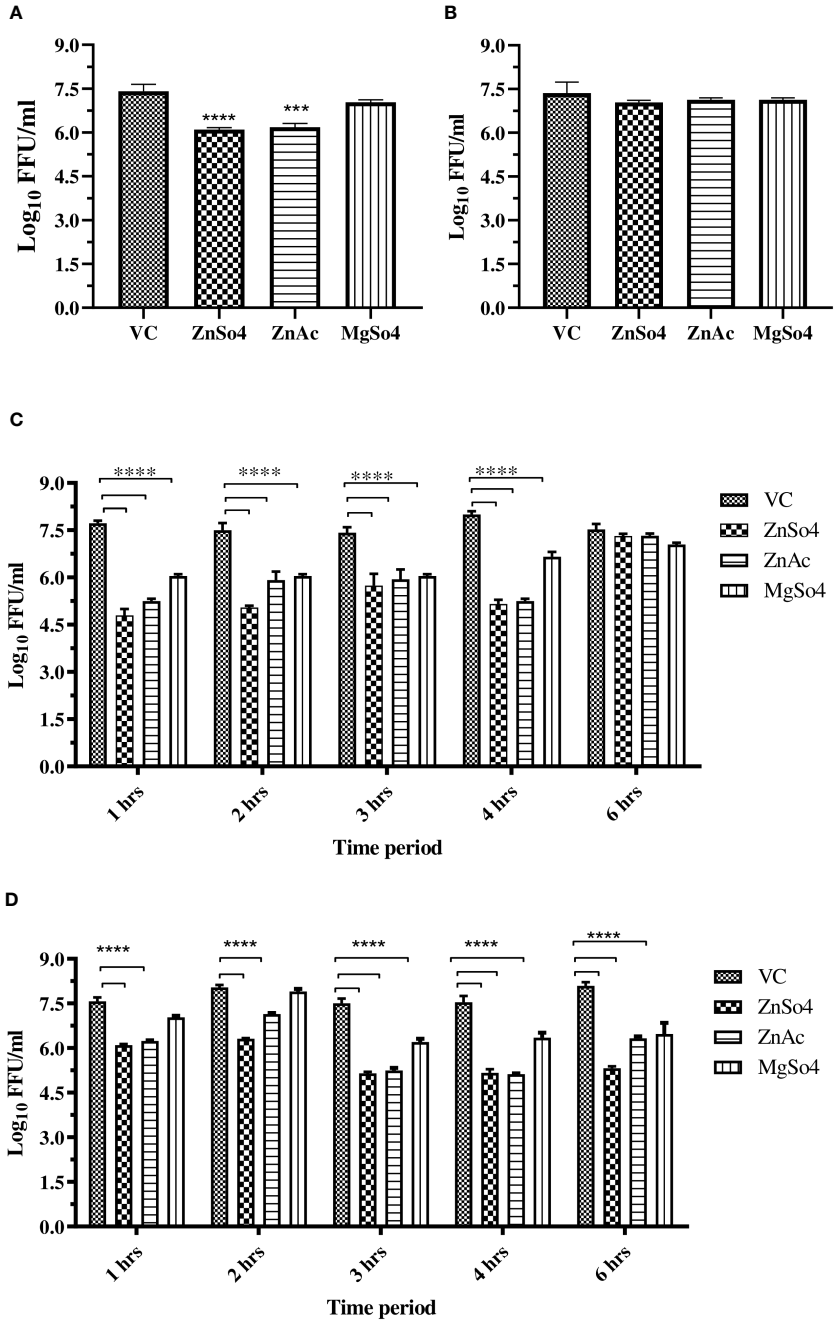

FIGURE 4
(A) Entry assay, and **(B)** Attachment assay, **(C)** Effect of time of addition of zinc and magnesium salts to CHIKV infected Vero CCL-81 cells on virus titre. **(D)** Effect of exposure time (time of removal) of zinc and magnesium salts to CHIKV infected Vero CCL-81 cells on virus titre. The experiments were conducted in triplicate and independently repeated twice. The virus titre was represented as mean $\log_{10}$ FFU/ml ± SE. All treatment groups were compared against the control group (VC) consisting of infected cells without treatment. **** p<0.0001.

assay was performed. The culture filtrates were investigated for the presence of genomic RNA, negative-strand RNA, which is a marker of viral replication, and infectious virus titre. A significant decrease in genomic RNA was observed in the cultures treated with zinc and magnesium salts (Figure 5A). Compared to transfected cultures that did not receive any treatment, a lower copy number of CHIKV negative strand RNA was observed in cultures treated with all the salts studied. However, the effect was more pronounced in cultures treated with zinc sulphate, followed by zinc acetate and magnesium sulphate (Figure 5B). A significant decrease in the infectious virus titre was noticed in cultures treated with zinc and magnesium salts, and the effect was more pronounced in cultures that received zinc sulphate treatment (Figure 5C). The results suggest that zinc salts affect the post-entry stages.

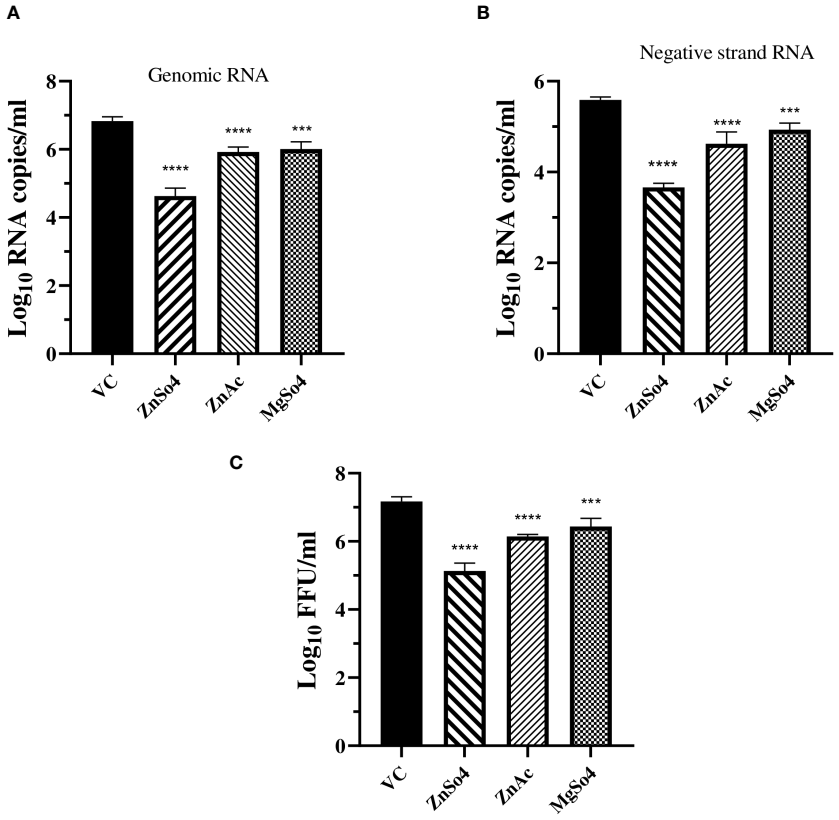

FIGURE 5
Effect of zinc and magnesium salts on the production of genomic RNA **(A)**, negative strand RNA **(B)**, and infectious virus particles **(C)**, when the cells were transfected with viral RNA. The virus output was expressed as mean log $_{10}$ RNA copy number or FFU/ml ± SE One-way ANOVA test was used to compare the differences between treated and untreated cultures with statistical significance indicated as *** p<0.001; **** p<0.0001.

3.9 Treatment of infected mice with zinc and magnesium salts decreased viral load in serum

Oral feeding (30 mg/kg) of CHIKV-infected mice with zinc sulphate, zinc acetate, and magnesium sulphate significantly reduced the viral RNA load in serum. At 2nd dpi, magnesium sulphate-treated groups showed one $\log_{10}$ reduction (99% reduction); at 3rd dpi, all the treatment groups showed 2.1 $\log_{10}$ reduction (99.2% reduction) of viral RNA compared to animals (VC), which did not receive the salts ($p < 0.05$). At 5th dpi, the reduction in viral RNA copy number was 1.8 $\log_{10}$ ($p < 0.05$). At 7th dpi, no difference in the viral RNA load was observed between treated and untreated animals. The therapeutic effect was more pronounced for magnesium sulphate on the 2nd day, while the effects of both zinc and magnesium salts were the same from the 3rd day onwards (Figure 6).

3.10 Molecular interaction of zinc and magnesium ions with CHIKV proteins

Molecular docking of CHIKV proteins with zinc and magnesium ions was performed using the MIB2 server. Docking results of zinc ions with the envelope proteins suggested strong interaction with the conserved residues of the E1 domain including His3, Asp75, Asn100, His152, His230, and His331 (Figure 7A). Moreover, the results showed that zinc ions bound best to the CHIKV nsP2 protease and magnesium ions bound best to the helicase domain compared to other structural and non-structural proteins. The highest possible interaction of Zn ions involved the active site amino acid residues Cys448, Glu520, His548, and Asp550 (Figure 7B), and the highest possible interaction of Mg ions involved the residues Tyr132, Asp135, Asp137, and Glu138 (Figure 7C). The detailed binding score and interacting residues are described in Supplementary Table 1.

4 Discussion

The present study was carried out to explore the anti-chikungunya activity of zinc and magnesium salts. The results revealed the potential antiviral effects of zinc sulphate, zinc acetate, and magnesium sulphate against CHIKV *in vitro* and *in vivo*. The findings were confirmed by evaluating the titre of infectious virus particles using the FFU assay, the genomic viral RNA copy number and negative strand RNA copy number using qRT-PCR, and the percent of cells infected by immunofluorescence assay.

Earlier studies have shown that chelation of zinc, leading to its deficiency, induces oxidative stress, which specifically affects positive-

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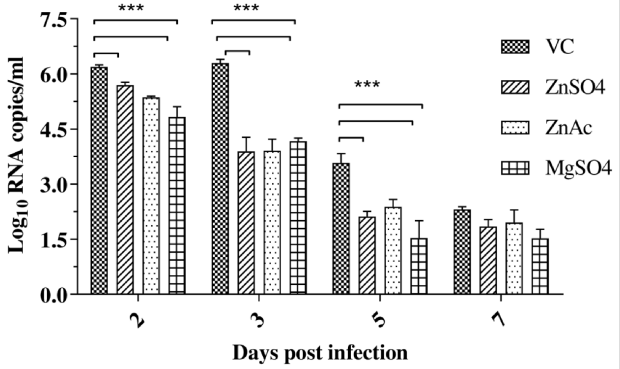

FIGURE 6
Effect of zinc sulphate, zinc acetate and magnesium sulphate (30mg/kg) administered orally to C57/BL6 mice on serum viral RNA load. The viral RNA copies were measured on the 2nd, 3rd, 5th, and 7th day after infection. The results are presented as $\log_{10}$ RNA copies/ml ± SE. Statistical significance was denoted as ***p<0.001.

strand RNA viruses, including CHIKV (Khan et al., 2021). Zinc ions have been reported to inhibit the pH-dependent liposomal fusion of the E1 domain of alphavirus envelope glycoproteins by binding with high affinity to histidine, cysteine, and asparagine residues (Bracha and Schlesinger, 1976). The entry assay revealed that zinc might interfere with virus entry and uncoating, a finding further supported by molecular interaction studies of zinc ions with E1 and E2 glycoproteins. Zinc ions exhibited high affinity for His3, His230, and His331, which are conserved in the alphavirus E1 domain and play a critical role in the fusion process. This affinity might have inhibited the fusion-infection process by competing for protonation of these histidine residues, similar to what was described for Semiliki forest virus (Corver et al., 1997).

In the present study, supplementation with zinc and magnesium salts affected CHIKV post-entry stages of virus life cycle. Zinc acetate inhibits the nSP2 protease activity of CHIKV (Saha et al., 2018), which was confirmed in the *in silico* experiments of the present study. The zinc ion forms a rigid coordination with Cys448, Asp538 and His548 with a bond length of <2.5A°, suggesting possible inhibition of the active site of the CHIKV protease. Similar results have been observed in many studies, suggesting zinc as a potent inhibitor of the nSP2 protease (Saisawang et al., 2015). Studies have reported that one cycle of CHIKV replication and virion formation takes 5–6 h post infection (Reis et al., 2022). TOA, TOR, and entry bypass studies showed that zinc and magnesium salts might have inhibited CHIKV post-entry stages, including proteolytic processing of polyproteins, replication, and assembly of virions, which required treatment initiation before 4 hours of post-infection. Though zinc salts reduced CHIKV titre even within one hour of exposure, the maximum reduction in virus titre was observed in cultures exposed for atleast three hours after infection. This observation suggests that zinc salts might act at multiple steps after infection including entry, proteolytic processing of polyproteins, replication, and assembly of virions. The inhibitory effect of zinc salts was more evident against the synthesis of negative strand RNA, indicating that zinc salts might affect replication or pre-replication stages such as proteolytic processing. Treatment after 4 hours of virus entry had no effect on virus titre, suggesting that zinc and magnesium salts may not affect the viral assembly and release. Among the zinc salts, zinc sulphate was more effective in inhibiting CHIKV compared to zinc acetate. Zinc sulphate (23% zinc) is generally more soluble, and bioavailability is high compared with other forms of zinc (Herman et al., 2002). The bioavailability of zinc acetate and zinc sulphate might be the same, but there is no human data available (Wegmüller et al., 2014). Even though both compounds release zinc ions, the rate at which these ions are released and their availability for cellular uptake can vary.

The finding that magnesium sulphate inhibits CHIKV is a serendipitous discovery, and the mechanism of antiviral activity is not clearly understood. The antiviral activity of magnesium sulphate against mouse hepatitis virus has been reported (Mizutani et al., 1994). *In silico*, docking of magnesium ions with CHIKV proteins revealed that it binds to the nSP2 helicase. However, it has been shown that Mg2+ ions are required for nSP1 methyl transferase and guanylylation activities and nSP2 helicase nucleoside triphosphatase function (Karpe et al., 2011; Kaur et al., 2018). At the same time, magnesium has been reported to have a role in ~600 enzymatic reactions (de Baaij et al., 2015). This suggests that magnesium sulphate might exert antiviral activity through host-dependent mechanisms rather than interfering with the activity of viral proteins. However, the findings from the different time dependent assays and the entry bypass assay suggest that magnesium salt effects only post-entry stages or during replication of CHIKV. Alternatively, high concentrations of magnesium might affect viral protein activity through feedback regulation. The antiviral activity post-infection also indicates that host mechanisms that function to suppress viral replication might be potentiated by magnesium ions. Magnesium sulphate might exert a distinct mechanism that may not involve direct interference with viral entry, and this needs further investigation.

In-vivo studies using C57BL6 mice revealed that oral feeding of infected mice with zinc and magnesium salts reduced the initial viral RNA load, though, at 7th dpi, the viral RNA copy numbers (~100 copies) were similar in treated and untreated mice. However, in treated mice, the reduction in viral RNA copy number was comparatively faster and reached ~100 copies in 5th dpi, while it occurred at 7th dpi in untreated mice, suggesting that feeding with zinc and magnesium salts might lead to rapid clearance of viruses, thereby reducing inflammation and subsequent sequelae. The main limitation of the *in-vivo* study is that the mouse model used in the study did not develop any clinical symptoms to investigate the effect of salts on the clinical course of the disease, including arthritis. Further the studies on inflammatory response in the infected mice need to be conducted.

Plasma zinc levels range between 9 and 20 µmol/L. The human zinc half-life is approximately 280 days (Nriagu, 2011). According to the Toxnet database of the U.S. National Library of Medicine, the oral LD50 for zinc, measured in rats and mice, is close to 3 g/kg body weight (Coni et al., 2021). In the present study, zinc concentrations ranging from ~17 to 45 µmol are required for a 50% reduction in the virus titre *in-vitro* when added at different time points. This concentration mimics the normal plasma concentration. The safety profile of high-dose intravenous zinc (HDIVZn) has been well documented in the literature. In the treatment of burns, HDIVZn has been administered at doses ranging from 26.4 to 37.5 mg/day for eight consecutive days

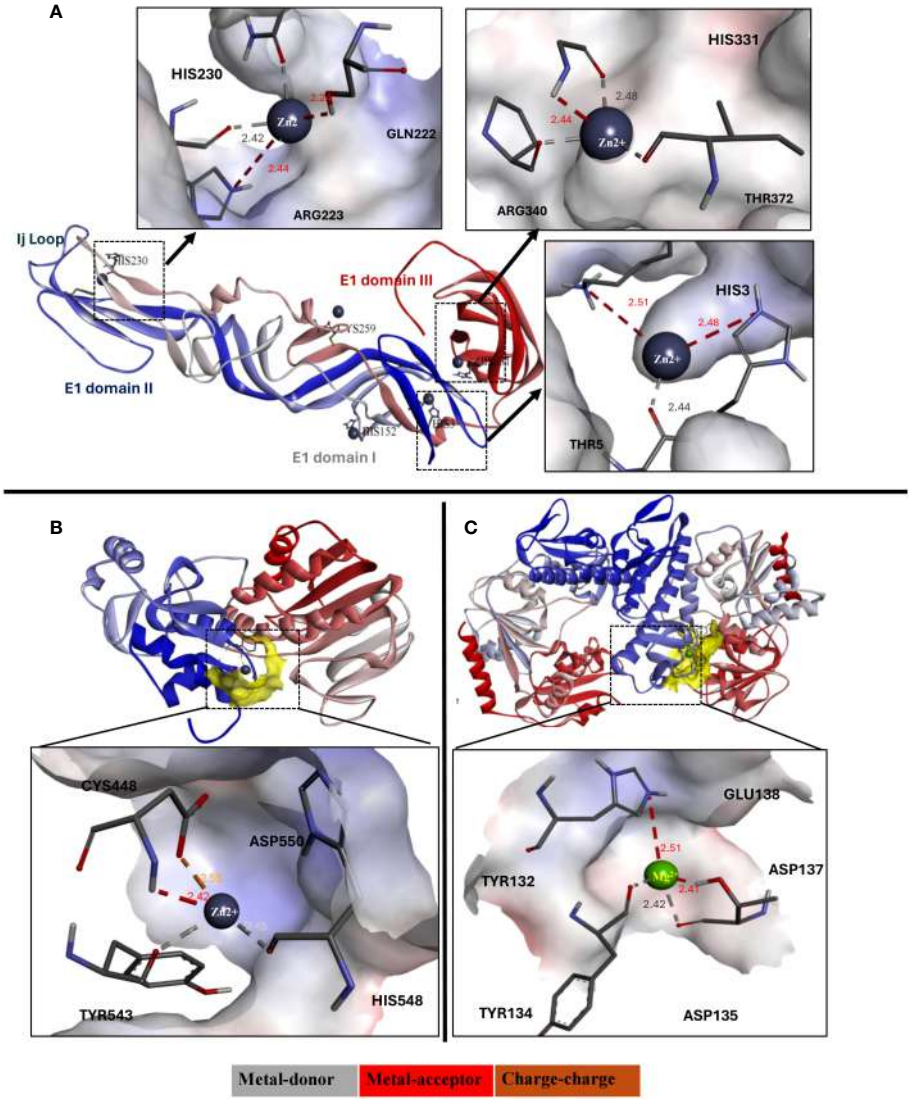

FIGURE 7
(A) Ribbon diagram (3d view) of E1 domain of CHIKV envelope glycoprotein interacting with zinc ions, and their detailed interaction and binding properties. **(B)** Ribbon diagram (3d view) of CHIKV protease interacting with zinc ions, and **(C)** Ribbon diagram (3d view) of CHIKV helicase interacting with magnesium ions. The ribbon structure of proteins depicted in blue to red color represents N-terminus to C-terminus sequence.

without any reported side effects (Berger et al., 2007; Cvijanovich et al., 2016). Notably, oral zinc doses exceeding 75 mg/day have demonstrated antiviral effects against common cold viruses, including influenza viruses (Hemilä, 2017). Mild adverse effects associated with zinc supplementation have been reported at dosages surpassing 200 mg/day (Fosmire, 1990).

5 Conclusion

Since viral persistence is one of the contributors to chikungunya-associated chronic arthritis, early treatment with zinc and magnesium salts might help in the clearance of the virus and prevent the subsequent development of arthritis. However, further trials in humans are needed. To conclude, the current study reports that zinc and magnesium salts exhibit anti-CHIKV activity, which might have implications for designing therapeutic strategies against CHIKV infection and the development of CHIKV-associated arthritis.

Data availability statement

The original contributions presented in the study are included in the article/Supplementary Material. Further inquiries can be directed to the corresponding authors.

Ethics statement

The animal study was approved by Committee for Control and Supervision of Experiments on Animals (CCSEA) (IAEC number CHK 1501, approved on December 20, 2017). The study was conducted in accordance with the local legislation and institutional requirements.

Author contributions

KD: Formal analysis, Investigation, Visualization, Writing – original draft, Data curation, Methodology, Software, Validation. SS: Data curation, Formal analysis, Investigation, Methodology, Software, Validation, Writing – original draft. MK: Formal analysis, Methodology, Writing – review & editing. SC: Writing – review & editing, Investigation, Project administration, Resources, Software, Supervision. KA: Investigation, Project administration, Software, Supervision, Writing – review & editing, Conceptualization, Data curation, Formal analysis, Visualization, Writing – original draft. DP: Conceptualization, Formal analysis, Investigation, Project administration, Supervision, Visualization, Writing – original draft, Writing – review & editing, Funding acquisition, Resources.

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Conflict of interest

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fcimb.2024.1335189/full#supplementary-material

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PLOS | NEGLECTED TROPICAL DISEASES

Administration of E2 and NS1 siRNAs Inhibit Chikungunya Virus Replication In Vitro and Protects Mice Infected with the Virus

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Abstract

Background: Chikungunya virus (CHIKV) has reemerged as a life threatening pathogen and caused large epidemics in several countries. So far, no licensed vaccine or effective antivirals are available and the treatment remains symptomatic. In this context, development of effective and safe prophylactics and therapeutics assumes priority.

Methods: We evaluated the efficacy of the siRNAs against ns1 and E2 genes of CHIKV both *in vitro* and *in vivo*. Four siRNAs each, targeting the E2 (Chik-1 to Chik-4) and ns1 (Chik-5 to Chik-8) genes were designed and evaluated for efficiency in inhibiting CHIKV growth *in vitro* and *in vivo*. Chik-1 and Chik-5 siRNAs were effective in controlling CHIKV replication *in vitro* as assessed by real time PCR, IFA and plaque assay.

Conclusions: CHIKV replication was completely inhibited in the virus-infected mice when administered 72 hours post infection. The combination of Chik-1 and Chik-5 siRNAs exhibited additive effect leading to early and complete inhibition of virus replication. These findings suggest that RNAi capable of inhibiting CHIKV growth might constitute a new therapeutic strategy for controlling CHIKV infection and transmission.

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Introduction

Chikungunya virus (CHIKV) is a mosquito-transmitted alphavirus belonging to the family *Togaviridae*. CHIKV is responsible for an acute infection, characterized by high fever, arthralgia, myalgia, headache, and rash [1,2,3]. Although having immense medical importance, effective vaccine or specific therapy is not commercially available. Currently, strict attention is given to good infection control practices that emphasizes mosquito control program.

RNA interference (RNAi) is the process of sequence-specific, post-transcriptional gene silencing (PTGS) in animals and plants, which is induced by 21- to 23-nucleotide (nt) small interfering RNA (siRNA) that is homologous in sequence to the silenced gene [4,5,6]. RNAi not only regulates gene expression in the mammalian cells but also acts as a cellular defense mechanism against the invaders, including the viruses. In recent years, inhibition of specific genes by siRNAs has proven to be a potential therapeutic strategy against viral infection. For instance, inhibition of virus replication and gene expression by directly introducing siRNAs into the cells have been reported for several RNA viruses, including several important human pathogens, such as poliovirus, HIV, Hepatitis, Chandipura and influenza virus [7–24]. It has been also shown that alphaviruses such as Semliki Forest virus [7], Venezuelan equine encephalitis [20], O'nyong-nyong virus [14] are susceptible to small interfering RNA action. Recently Dash et al., [22] have demonstrated that introduction of exogenous siRNAs can inhibit replication of CHIKV *in vitro*. The success of this study is limited as siRNAs used against ns3 and E1 genes of CHIKV were shown to reduce replication by 65% by 48 h p.i. and not evaluated in-vivo [22]. Cell clones expressing shRNAs against CHIKV E1 and nsP1 genes showed significant inhibition of CHIKV replication as compared to the scrambled shRNA cell clones and non-transfected cell controls [25].

Alphaviruses contain a linear, positive sense, single stranded RNA genome of approximately 11.8 kb. RNA genome consists of a capped 5′ non-coding region (NCR) and 3′ polyadenylated NCR. The non-structural proteins, nsP1, nsP2, nsP3 and nsP4 are required for the virus replication; the structural proteins, which consist of capsid and envelope proteins (E1, E2, E3 and 6K), are synthesized as polyproteins and are cleaved by capsid autoproteinase and signalases [26]. Given the similarity of the CHIKV genomic structure to those of other alphaviruses, CHIKV is expected to encode spikes on the virion surface that is each formed by three E1–E2 heterodimers where the E1 glycoproteins mediate the fusion and the E2 glycoproteins interact with the host receptor [26,27,28]. Nsp1 protein is involved in the RNA synthesis and capping. E2 and ns1 genes are highly conserved in CHIKV strains

Author Summary

Despite having immense medical importance, still vaccine, chemoprophylactic, or effective therapeutic measures are not commercially available for chikungunya. Only strict attention to good infection control practices may prevent CHIKV infection. The pathogenic properties of CHIKV necessitate the development of an efficient antiviral therapies. Four siRNAs each, targeting the E2 and ns1 genes of chikungunya were designed and evaluated for their efficiency in inhibiting CHIKV growth in *in vitro* and *in vivo* model systems. Efficiency of these siRNAs in controlling CHIKV replication *in vitro* and *in vivo* was assessed by the real time PCR, IFA and plaque assay. Chik-1 and Chik-5 siRNA ids efficiently inhibited CHIKV replication in the virus-infected Vero-E6 cells and mice. CHIKV replication was completely inhibited in the virus-infected mice when administered 72 hours post infection (p.i.). The combination of Chik-1 and Chik-5 siRNAs exhibited additive effect leading to early and potent inhibition of virus replication. Taken together, these findings suggest the promising efficacy of RNAi ids in silencing sequence-specific genes of CHIKV and might constitute a new therapeutic strategy for controlling the CHIKV infection and transmission.

and are important in the entry and the multiplication in the host cell and, therefore, represent the rational targets for antiviral therapy.

In the current study, based on consensus sequence of CHIKV strains, the siRNA were designed to target the conserved regions in the E2 and ns1 genes of CHIKV. The efficacy of siRNAs targeted against E2 and ns1 genes individually or in combination in inhibiting the replication of CHIKV were evaluated *in vitro* (Vero cells) and *in vivo* (mice).

Materials and Methods

Ethics statement

All animals were handled in strict accordance with good animal practice as defined by Institutional Animal Ethics Committee (IAEC). The experiments were done in a biosafety level-2 animal facility at the National Institute of Virology. All animal work was approved by the IAEC. Animal experiments were carried out in strict compliance with Committee for the Purpose of Control and Supervision of Experiment on Animals (CPCSEA) guidelines, India.

Animals and route of siRNA delivery

Swiss albino and C57BL/6 mice (3–4 wks old; 20–25 grams) were maintained in the BSL-2 facility with controlled temperature (22°C), humidity, and a 12 h light/dark cycle. Mice received the CHIKV via one of three delivery methods: 1) Intra nasal (i.n.) 100 µl, 2) standard intra venous tail vein injection (i.v.) 200 µl, 3) Intra muscular injection (i.m.) 200 µl. siRNA (~20–25 µg/mouse) mixed with Hiperfect transfection reagent (Qiagen, Germany) and PBS (final volume 200 µl) via i.v. delivery method.

Vero E6 cells and virus strains

African Green monkey kidney (Vero-E6) cells were maintained in minimum essential medium with 10% fetal bovine serum, 100 U/mL penicillin, 100 µg/mL streptomycin and Neomycine 50 µg/mL. Vero-E6 cells grown under similar conditions were used for the propagation of CHIKV (African genotype, Strain No. 061573; Andhra Pradesh 2006; Accession Number EF027134), Dengue-2 (DENV-2) (Trinidad; TR1751) virus and Chandipura virus (CHPV) (Strain No. 034627; Andhra Pradesh; 2003) stock. CHIKV, DENV-2 and CHPV strains were obtained from virus repository of National Institute of Virology, Pune, India. Virus strains were passaged twice in Vero-E06 cells. Cell supernatants were harvested when 75% of the cells showed cytopathic effect, aliquoted, and stored at −80°C and used throughout the study. The virus stock titers were determined using real time PCR (8.26×10^8 CHIKV RNA copies/ml) and standard plaque assay (7×10^7 plaque-forming units/mL).

siRNA

CHIKV whole genome sequences were retrieved from GenBank NCBI database (http://www.ncbi.nlm.nih.gov) and consensus sequence was used to design the siRNA. All siRNAs were designed using HP OnGuard siRNA design (Table 1 and Fig. 1). siRNAs were then checked for the homology to all other sequences of the genome using non-redundant sequence database and the homology analysis tool. Four siRNAs each, targeting E2 and ns1 genes were designed and synthesized (Qiagen, Germany) (Table 1, Fig. 1). Negative control siRNA [ncsiRNA; siRNA against Chandipura virus (24) with no significant homology to any known mammalian gene was used as a non-silencing control in all RNAi experiments and were purchased from Qiagen, Germany. Fluorescent labeling of siRNA was performed using the Cy3 Silencer labeling kit (Ambion, USA) and modified as described in the manufacturer's protocol.

CHIKV infection and transfection

Vero E6 cells were infected with CHIKV (Multiplicity of infection MOI 5. Two h post infection (p.i.), cells were transfected with E2 (Chik-1, Chik-2, Chik-3, Chik-4), ns1 (Chik-5, Chik-6, Chik-7, Chik-8) siRNA and control using the Amaxa Nucleofector device II (Amaxa biosystems). After electroporation, Vero-E6 cells were incubated at 37°C until analyzed for inhibition of CHIKV replication. Cells were harvested at 24, 36 and 48 h p.i. and inhibition of CHIKV replication was determined by quantitative RT-PCR (qRT-PCR), plaque assay and ImmunoFluorescnce Assay (IFA).

Optimization of siRNA concentration

At two hours p.i. with CHIKV, Vero E6 cells were transfected with Chik-1, Chik-5 and combination of both siRNAs at different concentration (10, 50, 100, 150 and 200 pmol). After 24 h post transfection, total RNA was isolated from the tissue culture supernatant and cells. One step qRT-PCR was carried out to evaluate the inhibitory effect of siRNA.

MTT assay. Cytotoxicity tests were performed with Vero E6 cells using an in vitro toxicology assay kit (TOX-1, Sigma) based on the reduction activity of methyl thiazolyl tetrazolium (MTT). Twenty-four hours before transfection, 5×10^3 cells were seeded in a 96-well plate. The transfection was performed using Hiperfect transfection reagent according to the manufacturer's instructions. The cells were divided into four groups: 1) control group (No Hiperfect and siRNA); 2) Hiperfect group; 3) Chik-1 siRNA group; 4) Chik-5 siRNA group; and 5) Combination of Chik-1+Chik-5 siRNA (Comb-siRNA) group. Transfection of siRNAs was carried out on the following day as described earlier in the CHIKV infection and transfection section. Experiments were conducted with non-transfected or siRNA transfected (100 pmol) cells at 24 and 48 hr post-transfection. 24 hours after the siRNA transfection, the cells of the appropriate groups were subjected to MTT assay (TOX-1, Sigma). Blue formazon, solubilized by adding MTT solubilization solution to the wells, produced by

Table 1. Nucleotide sequences of siRNA designed for CHIKV genes.

No.	Si RNA Name	Location on genome (nt number)	Location on gene (nt number)		5′ Sequence 3′
E2 gene					
1	Chik-1	8574–8591	30–47	Sense	r (GGA CAA CUU CAA UGU CUA U) dTdT
				Antisense	r (AUA GAC AUU GAA GUU GUC C) dTdT
2	Chik-2	8955–8973	411–429	Sense	r (CCA CGA CCC UCC UGU GAU A) dTdT
				Antisense	r (UAU CAC AGG AGG GUC GUG G) dTdG
3	Chik-3	8848–8866	304–322	Sense	r (GGA ACA AUG GGA CAC UUC A) dTdT
				Antisense	r (UGA AGU GUC CCA UUG UUC C) dAdG
4	Chik-4	9386–9404	842–860	Sense	r (CCA CCG UGA CGU ACG GGA A) dTdT
				Antisense	r (UUC CCG UAC GUC ACG GUG G) dGdG
ns1 gene					
5	Chik-5	1641–1659	1563–1581	Sense	r (GGU CGA AAU CGA CGU GGA A) dTdT
				Antisense	r (UUC CAC GUC GAU UUC GAC C) dTdG
6	Chik-6	695–713	617–635	Sense	r (GGC UAA GAA CAU AGG AUU A) dTdT
				Antisense	r (UAA UCC UAU GUU CUU AGC C) dTdT
7	Chik-7	1107–1125	1029–1047	Sense	r (CGG CAU CCU UGC UAC AGA A) dTdT
				Antisense	r (UUC UGU AGC AAG GAU GCC G) dGdT
8	Chik-8	290–308	212–230	Sense	r (GGA UGA UGU CGG ACA GGA A) dTdT
				Antisense	r (UUC CUG UCC GAC AUC AUC C) dTdC

doi:10.1371/journal.pntd.0002405.t001

viable cells was quantified in ELISA reader (Biorad, USA) at 570 nm after subtracting the background reading at 650 nm. The data were presented as the percentage of viable cell numbers in the siRNA treated and untreated control wells.

In vivo inhibition of CHIKV using Chik-1 and Chik-5 siRNA

Swiss albino and C57BL/6 mice (3–4 weeks) were infected with approximately 1×10^6 pfu of CHIKV (100 µl of 10^7 pfu/ml; $\sim4.5\times10^8$ RNA copies/ml) by three routes viz.; i.v., i.n. and i.m. and RNA copies were checked in muscles on 2^{nd}, 4^{th}, 7^{th} and 14^{th} day p.i.. C57BL/6 mice (4–6 weeks) were infected by CHIKV (100 µl of 10^7 pfu/ml; $\sim4.5\times10^8$ RNA copies/ml) and CHIKV RNA copies were measured daily in the blood and muscles by one step qRT-PCR for fourteen days. siRNAs were complexed with HiPerfect^{TM} (QIAGEN, Valencia CA) according to the manufacturer's instructions and ~25 µg/mouse (1 mg/Kg body wt) was administered i.v. once after 48 or 72 h p.i.. Chik-1 siRNA, Chik-5 siRNA and combination of Chik-1 and Chik-5 siRNAs (Comb-siRNA) were used in different mice groups. Blood (~200 µl) was collected from siRNA, ncsiRNA, or saline injected mice groups at 0, 1, 2, 3 and 4 day post treatment. CHIKV E3 RNA was quantitated from the sera using qRT-PCR. In C57BL/6 mice, the 72 h time point was chosen for siRNA treatment. Blood and hind limb muscle tissues were harvested from C57BL/6 mice at 0, 1, 2, 3 and 4 day post siRNA injection. The tissues were dissected, weighed, crushed and macerated in liquid nitrogen using mortar pestle, and used for the RNA isolation.

Quantitative RT-PCR

RNA from the Vero-E6 cells, serum and mice tissues was extracted using QIAmp viral RNA minikit (QIAGEN, Valencia, CA) and trizol (Invitrogen USA) method respectively following the manufacturer's instructions. Viral load in serum and/or tissue samples were determined by absolute quantification using the

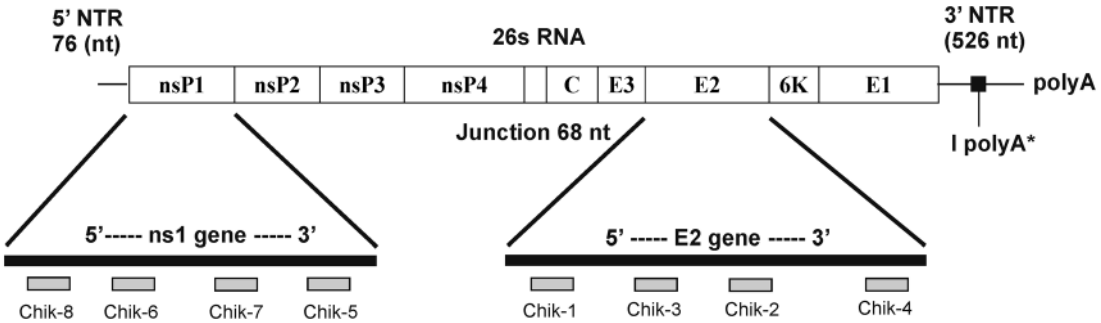

Figure 1. Schematic representation of the sites of the eight siRNA target sequence in CHIKV genome. (Exact location on CHIKV genome is depicted in Table 1.)
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standard curve method. One step RT-PCR was performed in 25 µl reaction mixture containing 5 µl RNA, 12.5 µl TaqMan One-Step RT-PCR 2× Master Mix, 1 µl 40×(RT+RNAasin) (Applied Biosystems) each 1 µl sense (µM), 1 µl anti-sense (µM) primer and 1 µl TaqMan probe. Primers were selected from the E3 structural protein region. Real-time one step RT-PCR was performed in a 96-well format using 7300 real time PCR system and SDS software V 1.0.2 (Applied Biosystems). The amplification program included: reverse transcription at 48°C for 30 min, initial denaturation at 95°C for 10 min, and 50 cycles of denaturation (95°C for 15 sec) and annealing and extension (60°C for 1 min). After the amplification, a melting curve was acquired to check the specificity of PCR products. A standard curve was generated by the amplification of serial dilutions of in vitro transcribed RNA of CHIKV (10^8 to 10^2 serial dilutions). After generation of standard curve, we compared unknowns to the standard curve and extrapolated the value. Viral titers were expressed as RNA copies per ml of serum or per ml of tissue culture suspension or per well or per mg tissue. Detection limit of real time PCR was 10 copies per reaction.

Plaque assay

Serial dilutions of tissue culture supernatants of infected and siRNA transfected cells were added to a monolayer of Vero E6 cells and the plates were incubated at 37°C for 1 h. After the incubation, the medium was replaced by overlay medium (2× MEM, 2% CMC and 10% FBS (Gibco, USA). The plates were incubated at 37°C for 72 h and the cells were stained with amido black and the plaques were counted.

Immune sera preparation against the CHIKV genotypes in mice

Groups of 3–4 weeks old swiss albino mice were inoculated intra-peritonealy with the CHIKV African strain (1:1 vol:vol mixture of CHIKV and Freund's complete adjuvant) and were maintained under standard laboratory conditions. Two booster doses with CHIKV along with Freund's incomplete adjuvant (1:1) were administered at weekly intervals. Blood samples were collected at pre and post immunization (7 days after the last dose). IgG antibodies were then purified using protein A column (Merck Biosciences, India) according to the instructions of the manufacturer.

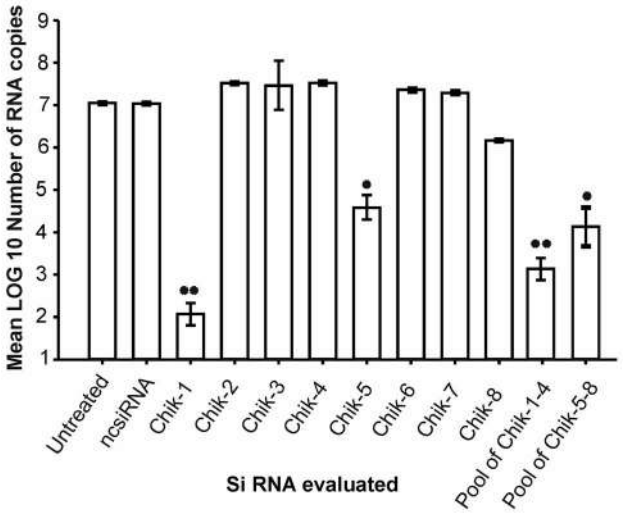

Figure 2. Effect of different siRNAs on CHIKV replication. Vero E6 cells seeded in 6 well plates were infected with CHIKV and after 2 h p.i., transfected with different siRNAs (100 pmol). Total RNA was isolated 24 h after virus infection. Amount CHIKV RNA was detected by measuring E3 RNA copies by real-time RT-PCR. siRNA Chik-1 and Chik-5 showed substantially stronger inhibition of CHIKV as compared to other siRNAs. Values are given as mean $\log_{10}$RNA copies/well ± SD. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$.
doi:10.1371/journal.pntd.0002405.g002

Immunofluorescence assay

Immunofluorescence assay (IFA) was carried out as described by Sudeep et al. (29). Vero E6 cells were fixed with acetone and blocked with 2% BSA in the phosphate buffered saline (pH 7.4) for 1 h. The cells were incubated with (1:100) mouse anti CHIKV antibody followed by incubation with FITC-conjugated rabbit anti-mouse (1:500) antibodies (Invitrogen, USA). Cells were counter stained with Evan's blue for one min. The slides were visualized using fluorescence microscope (Nikon eclipse T2000S and Q capture pro 5.0 software). Negative controls were similarly processed using pre-immune sera.

Histopathology

Hind limb tissues, excluding the femur bone, were fixed in 4% formaldehyde and were embedded in the paraffin. Thin section of 8 µm size were prepared. Tissues were stained with haematoxylin and eosin. Histopathological evaluation was performed on the muscle tissues of the hind legs from the control (saline injected, ncsiRNA), CHIKV infected (4, 5, 6 and 7 day p.i.), treatment groups (Chik-1 siRNA, Chik-5 siRNA and Comb-siRNA). siRNA treatment was given on third day p.i. and the tissues were harvested at 4, 5, 6 and 7 days p.i. and evaluated for necrosis, inflammation, regeneration, mineralization, fibrosis and the edema. Concurrently IFA was carried out to check the presence of CHIKV. Immunofluorescence assay (IFA) was carried out as described by Sudeep et al. [29]. The slides were incubated with (1:100) mouse anti CHIKV antibody followed by incubation with Alexa flor 546-conjugated rabbit anti-mouse (1:200) antibodies (Invitrogen USA). Cells were counter stained with DAPI for 10 seconds. The slides were visualized using fluorescence microscope (Nikon eclipse T2000S and Q capture pro 5.0 software). Negative controls were processed similarly.

Interferon gene expression analysis employing qPCR

For real-time reverse transcription RT-PCR analysis, hind limb muscle tissues were crushed in liquid nitrogen. RNA was extracted by using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. One step RT-PCR was performed using Quantitect SYBR Green RT PCR kit (Qiagen, Germany). Real-time PCR analysis used the following nucleotide primers: 5′-GGCCGAGGACTTTGATTGCACATT-3′ and 5′- AGGATGGCAAGGGACTTCCTGTAA-3′ for actin beta, 5′- AGGAGGAGTTTGATGGCAACCAGT -3′ and 5′- TCCTCATCCCAAGCAGCAGATGAA-3′ for Interferon alpha (INF-α) (NM_010502), 5′- TGTGGCAATTGAATGGGAGGCTTG-3′ and 5′- TCTCATAGATGGTCAATGCGGCGT -3′ for interferon beta (IFN-β), and 5′-AGCGGCTGACTGAACTCAGATTGT-3′ and 5′- ACTGCTTTCTTTCAGGGACAGCCT-3′ for interferon gamma (IFN-γ) (NM_008337). The 25-µl amplification reaction mixture contained 500 ng total RNA, 0.5 µM each primer pair, 0.25 of reverse transcriptase enzyme and 12.5 µl of 2× SYBR green qPCR Supermix (Qiagen, Germany). Cycling conditions were as follows: one cycle of 50°C for 30 min and one cycle of 95°C for 15 min followed by 45 cycles at 94°C for 15 s, 57°C for 30 s, 72°C for 30 s and 68°C for 15 s. The real-time PCR was performed by using a Rotor-Gene 3000 PCR

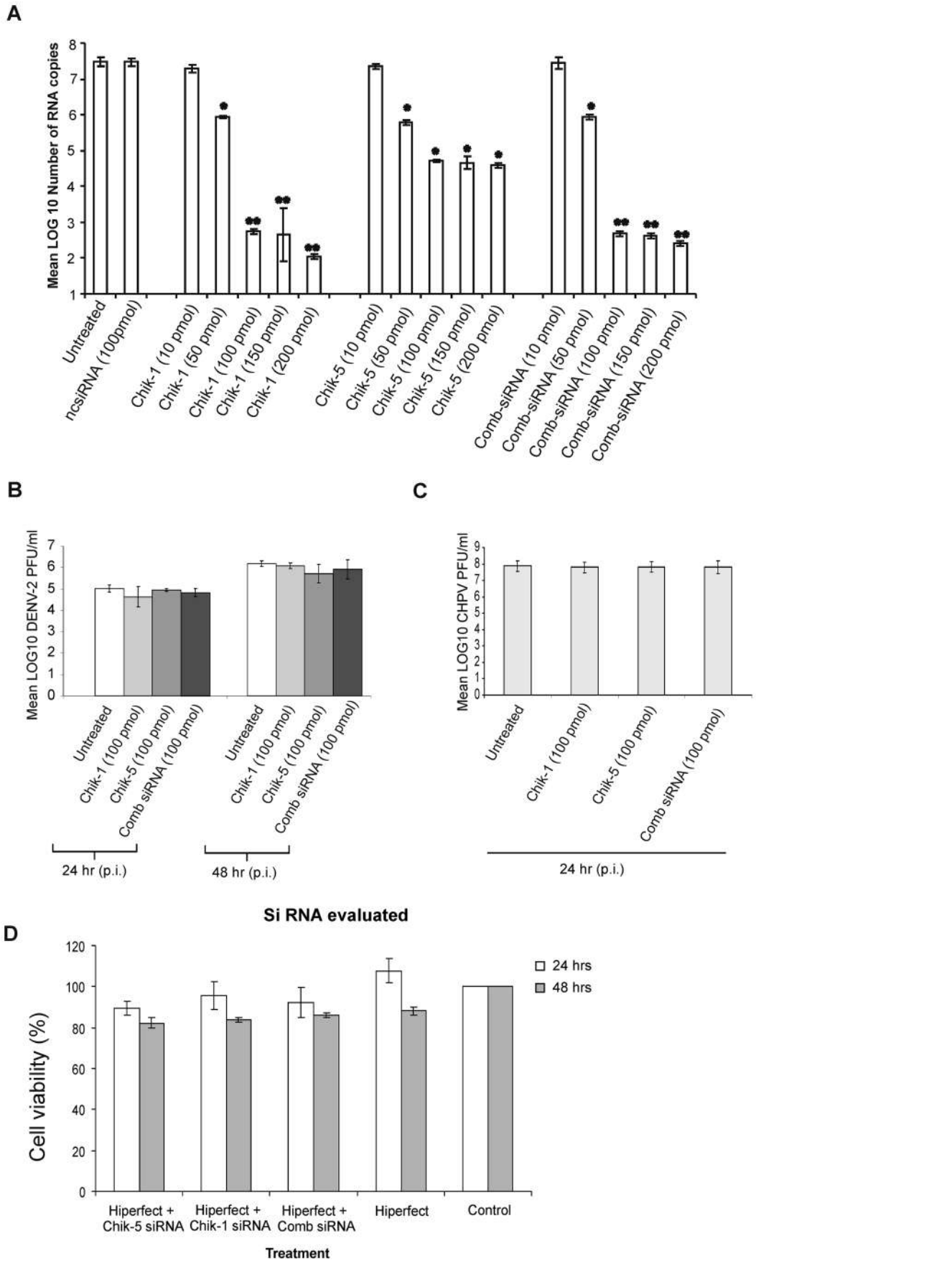
A
Mean LOG 10 Number of RNA copies
Untreated
ncsiRNA (100pmol)
Chik-1 (10 pmol)
Chik-1 (50 pmol)
Chik-1 (100 pmol)
Chik-1 (150 pmol)
Chik-1 (200 pmol)
Chik-5 (10 pmol)
Chik-5 (50 pmol)
Chik-5 (100 pmol)
Chik-5 (150 pmol)
Chik-5 (200 pmol)
Comb-siRNA (10 pmol)
Comb-siRNA (50 pmol)
Comb-siRNA (100 pmol)
Comb-siRNA (150 pmol)
Comb-siRNA (200 pmol)
B
Mean LOG10 DENV-2 PFU/ml
Untreated
Chik-1 (100 pmol)
Chik-5 (100 pmol)
Comb siRNA (100 pmol)
24 hr (p.i.)
48 hr (p.i.)
C
Mean LOG10 CHPV PFU/ml
24 hr (p.i.)
D
Si RNA evaluated
Cell viability (%)
24 hrs
48 hrs
Hiperfect + Chik-5 siRNA
Hiperfect + Chik-1 siRNA
Hiperfect + Comb siRNA
Hiperfect
Control
Treatment

Figure 3. Optimization of siRNA concentration. A) Vero E6 cells were transfected with Chik-1 and Chik-5 siRNAs individually as well as in combination at different concentration (10, 50, 100, 150 and 200 pmol). Total RNA was isolated 24 h after virus infection. Amount of CHIKV RNA was detected by measuring E3 RNA copies by real-time RT-PCR. siRNA Chik-1 and Chik-5 treatment at a concentration of 10 pM failed to reduce CHIKV RNA copies. Whereas all other concentrations of Chik-1 and Chik-5 significantly reduced the viral RNA copies. Values are given as mean $\log_{10}$ RNA copies/well ± SD. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$. **B**) Effect of Chik-1 and Chik-5 siRNAs on DENV-2 growth: Vero-E6 cells were infected with DENV-2 and Chik-1 (100 pmol) CHIK-5 (100 pmol) and Comb-siRNA (100 pmol) transfected after 2 h p.i. and DENV-2 titers were measured by plaque assay. **C**) Effect of Chik-1 and Chik-5 siRNAs on Chandipura virus (CHPV) growth: Vero-E6 cells were infected with CHPV and Chik-1 (100 pmol) CHIK-5 (100 pmol) and Comb-siRNA (100 pmol) transfected after 2 h p.i. and CHPV titers were measured by plaque assay **D**) Effect of siRNA–Hiperfect complex treatment on survival of Vero-E6 cells was accessed by MTT assay. siRNAs were used in 100 pmol concentration.
doi:10.1371/journal.pntd.0002405.g003

machine. The data were analyzed with Rotor-Gene real-time analysis software. Each sample was analyzed in duplicate and normalized to actin beta mRNA. Expression changes in interferon alpha, beta and gamma genes in CHIKV infected group, CHIKV infected mice treated with Chik-1, Chik-5 and Comb-siRNA group, and the control mice with Chik-1, Chik-5 and Comb-siRNA treatment were investigated using real time PCR analysis. Mice were mock-infected with CHIKV and treated with siRNA on third day p.i. and then gene expression determined at days 4, 5, 6, and 7 p.i.. Three mice were used for each treatment and each time point.

Statistical analysis

All data were expressed as mean ± standard deviation. The viral loads were log-transformed for improvement of normality. Statistical significance was determined by Dunnet's test using ANOVA. A value of $p<0.05$ was considered statistically significant. Fold change was compared using one way ANOVA and the groups were also compared by nonparametric Kruskal-Wallis test for confirmation of results.

Results

Based on the consensus sequences of CHIKV genomes, four siRNAs each were designed to have an antisense strand complementary to the E2 and ns1 RNA. The sequences and the corresponding genomic positions are shown in Table 1 and Fig. 1 respectively.

Efficiency of different siRNAs in reducing CHIKV replication in vitro

For the initial comparison of antiviral activity of different siRNAs, Vero-E6 cells were infected with CHIKV and transfected with different siRNAs (Chik-1 to Chik-8) 2 h p.i.. Chik-1 and Chik-5 were the most effective siRNAs, suppressing CHIKV copies by 5 $\log_{10}$ ($p<0.001$) and ~2.5$\log_{10}$ ($p<0.05$) RNA copies respectively (Fig. 2). The pool of siRNAs Chik 1–4 (4 $\log_{10}$; $p<0.001$) and Chik5–8 (3 $\log_{10}$; $p<0.001$) did not increased the CHIKV suppression in Vero E6 cells (Fig. 2). Results obtained with the individual siRNAs and pool of siRNAs indicated that only siRNAs Chik-1 and Chik-5 possessed the antiviral activity against CHIKV. Therefore only Chik-1 and Chik-5 and Comb-siRNA were used for further studies. The reduction in the CHIKV copies by Chik-1 and Chik-5 was initiated at the siRNA concentrations of 50 pmol, and reached a plateau at 100 pmol (Fig. 3 A). Chik-1 and Chik-5 showed sequence dependent inhibition and showed no reduction in the dengue-2 (Fig. 3B) and the Chandipura virus (Fig. 3C) replication in Vero-E6 cells.

Effect of siRNA treatment on proliferation of VeroE6 cells (MTT assay)

Effect of Chik-1, Chik-5 and Comb-siRNAs (100 pmol) transfection on survival of Vero-E6 cells was assessed by the MTT assay. At 24 h, transfection of Chik-1 siRNA (95.1±6.51), Chik-5 siRNA (89.46±3.19), Comb-siRNA (92.11±7.11) and Hiperfect reagent (95.88±11.47) do not exhibited any significant change in proliferation of Vero E6 cells (Fig. 3D). At 48 h,

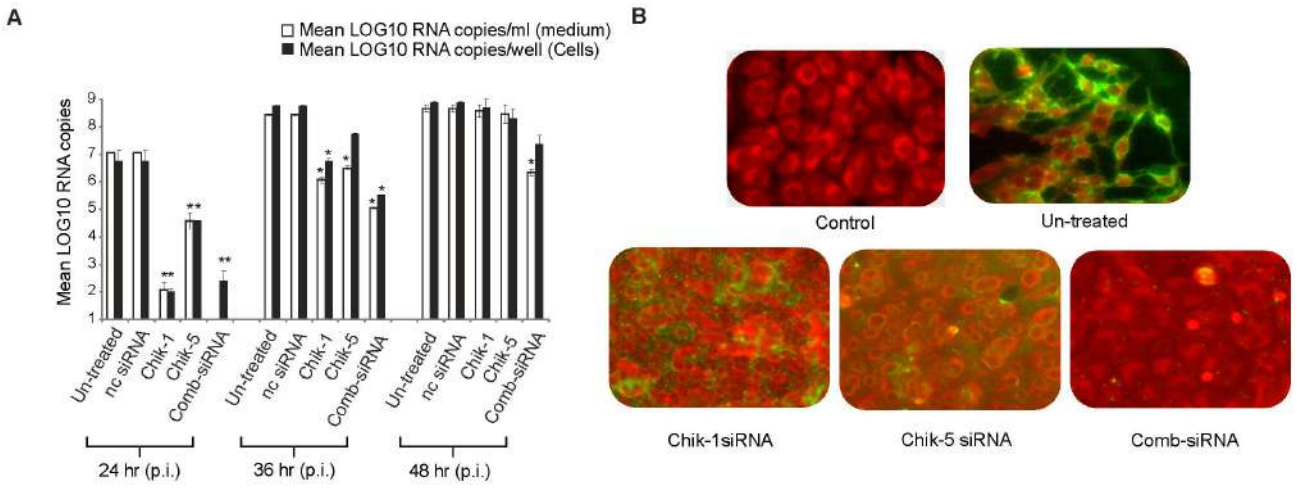

Figure 4. Evaluation of siRNAs directed against CHIKV E2 and ns1. A) Quantitative analysis of intra cellular and extra cellular CHIKV RNA copies using real time PCR: Total RNA was isolated at 24, 36 and 48 h after virus infection and E3 RNA copies determined by real-time RT-PCR. Values are given as mean RNA copies/well ± SD for cells and mean RNA copies/ml ± SD for culture medium. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$; ***$p<0.001$. **B**) Detection of CHIKV in Vero E6 cells using immuno-fluorescence microscopy: Vero E6 cells seeded in 6 well plates were infected with CHIKV and after 1 h transfected with 100 pmol of the indicated siRNAs. Cells were fixed 48 h later and CHIKV (green) were detected with fluorescein labeled antibodies. Cells were co-stained with Evan's blue (red) to visualize the cell morphology.
doi:10.1371/journal.pntd.0002405.g004

Table 2. Plaque assay showing inhibitory effect of siRNAs on CHIK virus replication in Vero E6 cell line at 48 h.

Material	Virus titer pfu/ml (SD)	Virus inhibition compared to untreated
Untreated	4×10^7 (1.9×10^7)	Nil
CHIKV+Chik-1 siRNA	2×10^2 (1.1×10^2)	>5 $\log_{10}$**
CHIKV+Chik-5 siRNA	4×10^4 (1.8×10^4)	3 $\log_{10}$*
CHIKV+Comb-siRNA	7.5×10^4 (6.3×10^4)	~3 $\log_{10}$*
CHIKV+ncsiRNA	4×10^7 (1.8×10^7)	Nil

Values are given as mean pfu/ml (SD).
Significance ANOVA, Dunnett's test:
*$p<0.05$;
**$p<0.01$.
doi:10.1371/journal.pntd.0002405.t002

transfection of Chik-1 siRNA (83.71±9.24), Chik-5 siRNA (82.13±2.71), Comb-siRNA (86.1±1.65) and Hiperfect reagent (88.02±2.58) displayed small reduction in viable cell number (Fig. 3D).

siRNA stability

Transfected Cyanine 3 dye labeled siRNAs showed signal at 4 h and 6 h whereas at 24 h signal was minimal, but still present compared to control treatment (Supplementary Information Fig. S1). Chik-1 and Chik-5 siRNAs were stable till 24 h.

Effect of Chik-1 and Chik-5 siRNAs on CHIKV replication

Figure 4A depicts the effect of Chik-1, Chik-5 and Comb-siRNAs on the CHIKV replication at different time points. At 24 h p.i., treatment of Chik-1 and Chik-5 siRNAs resulted in the reduction of 5 $\log_{10}$ and 3 $\log_{10}$ CHIKV RNA copies respectively in cells and the supernatant (Fig. 4A). At 36 h p.i., 3 $\log_{10}$ (Chik-1), and 2 $\log_{10}$ (Chik-5) reduction in CHIKV RNA copies was observed in tissue culture supernatant whereas 2 $\log_{10}$ reduction was recorded in cells with Comb-siRNAs (Fig. 4A). At 48 h p.i., no significant reduction in CHIKV RNA copies was noted in the cells and the supernatant. Overall, the siRNAs directed against E2 gene (Chik-1) were more efficient in inhibiting CHIKV replication than the siRNA directed against ns1 region (Chik-5). We further evaluated the additive advantage of treatment with Comb-siRNAs. In supernatant, 5 $\log_{10}$ ($p<0.001$), 2.5 $\log_{10}$ ($p<0.05$) and 2.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) reduction in CHIKV copies was observed at 24, 36 and 48 h respectively when compared to virus

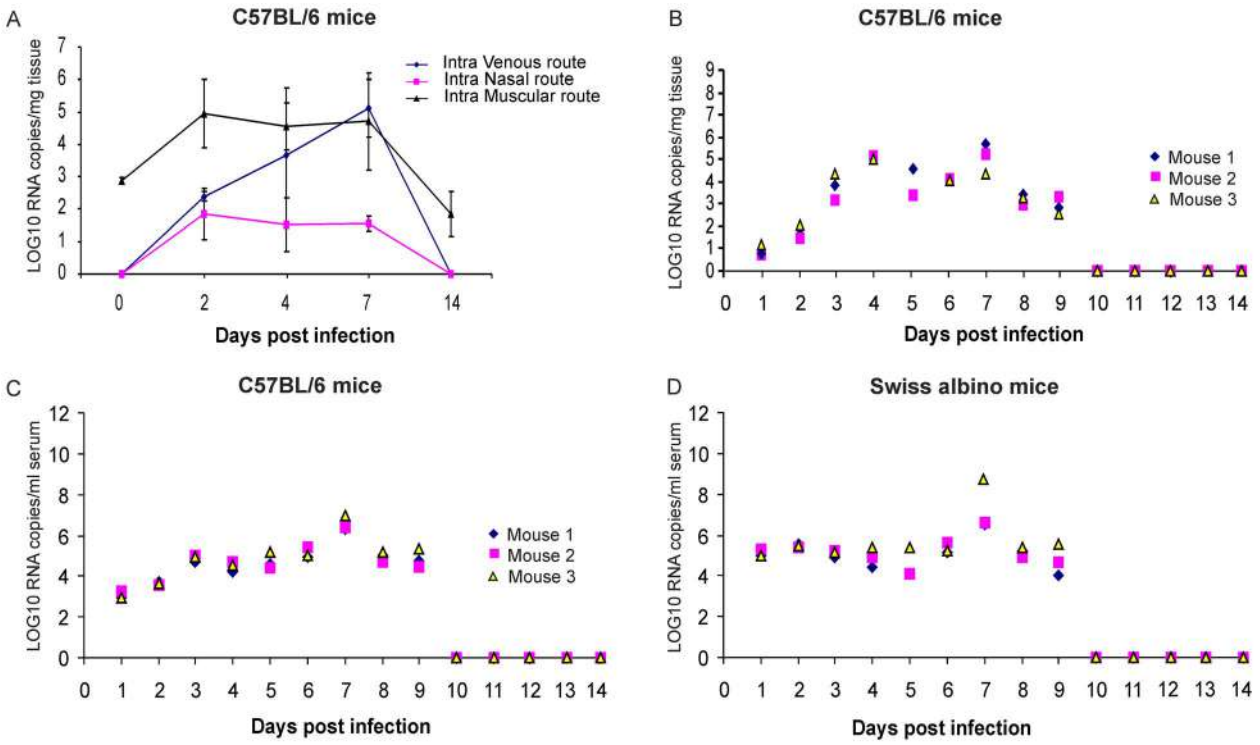

Figure 5. CHIKV replication in C57BL/6 and Swiss albino mice. C57BL/6 mice were infected with CHIKV (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) by three different routes viz. intra venous, intra nasal and intra muscular. **A**) CHIKV copies were measured in hind limb muscle tissues on 0, 2, 4, 7, and 14 days p.i.. **B**) CHIKV RNA copies in hind limb muscle tissues of C57BL/6 mice on 1–14 days p.i. **C**) CHIKV RNA copies in serum of C57BL/6 mice on 1–14 days **D**) CHIKV RNA copies in serum of swiss albino mice.
doi:10.1371/journal.pntd.0002405.g005

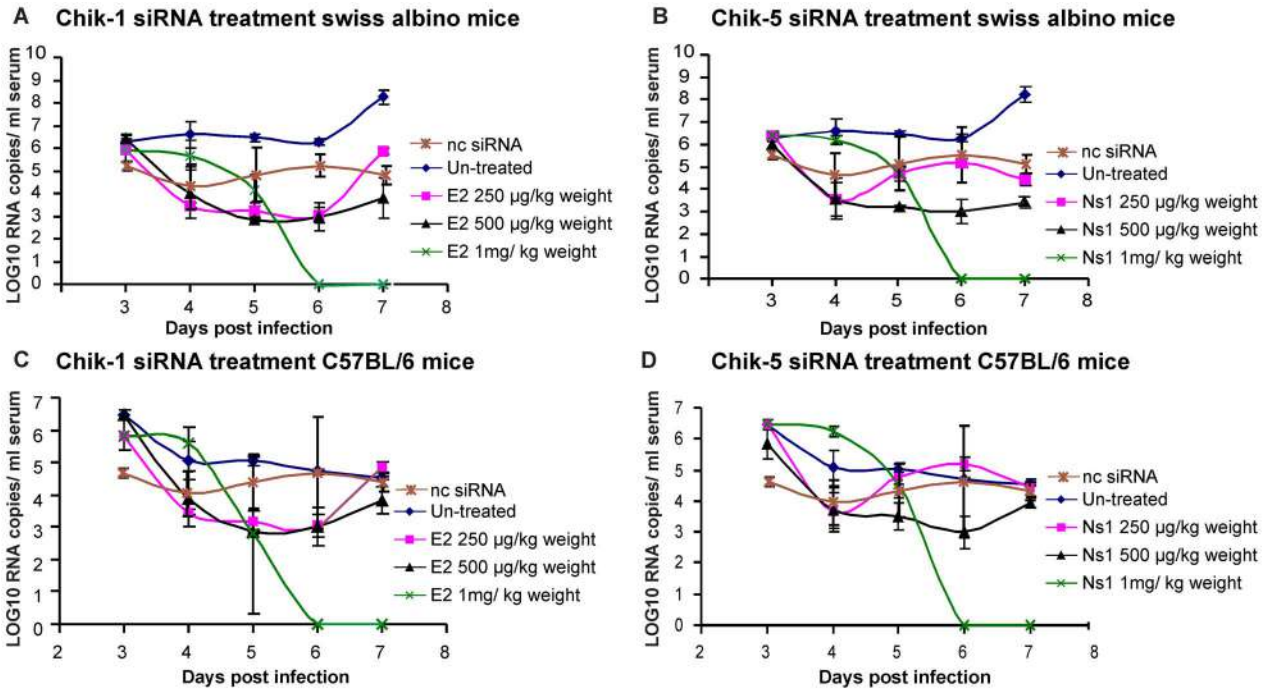

Figure 6. Dose dependent reduction in CHIKV copies/ml serum after injection with siRNA Chik-1 and Chik-5 in swiss albino and C57BL/6 mice infected with CHIKV. Swiss albino and C57BL/6 mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). After 72 h p.i. swiss albino mice were inoculated i.v. with **A**) Scrambled siRNA (siRNA against chandipura virus), 250 µg, 500 µg and 1 mg/kg body weight Chik-1 siRNA (n = 3 for each treatment and time point), **B**) Scrambled siRNA, 250 µg, 500 µg and 1 mg/kg body weight Chik-5 siRNA (n = 3 for each treatment and time point). After 72 h of p.i. C57BL/6 mice were inoculated i.v. with **C**) Scrambled siRNA, 250 µg, 500 µg and 1 mg/kg body weight Chik-1 siRNA (n = 3 for each treatment and time point), **D**) Scrambled siRNA, 250 µg, 500 µg and 1 mg/kg body weight Chik-5 siRNA (n = 3 for each treatment and time point). At indicated time after injection of siRNA blood was collected from eye and RNA was isolated from serum. CHIKV E3 RNA copies were quantitated using real time RT-PCR. Values are given as LOG10 RNA copies/ml serum.
doi:10.1371/journal.pntd.0002405.g006

infected control. In cells, 4.5 $\log_{10}$ ($p<0.001$), 3 $\log_{10}$ ($p<0.05$) and 2 $\log_{10}$ ($p<0.05$) reduction was obtained at 24, 36 and 48 h respectively. Importantly, the Comb-siRNA could prolong the inhibitory effect as compared to individual siRNAs (Fig. 4A).

When plaque assay was used as the measure of CHIKV replication, Chik-1 siRNA yielded a reduction of 5 $\log_{10}$ at 48 h p.i. (Table 2). Chik-5 reduced 3 $\log_{10}$ and Comb-siRNA showed reduction of 3 $\log_{10}$ in the virus titer. At 24 and 36 h p.i., cytopathic effects were not observed in the treated cultures where as commencement of cytopathic effects was observed in the untreated control from 24 h p.i. demonstrating the inhibitory effect of the siRNAs. These results were consistent with our real time RT-PCR results and the plaque assay results, IFA also showed the reduction of viral antigen in Chik-1 and Chik-5 siRNAs treated cells (Fig. 4 B).

Swiss albino and C57 BL/6 mice are permissive to CHIKV infection

As human muscle cells are the target of CHIKV infection, we evaluated the i.m. route along with i.v. and i.n. route for CHIKV infection. Infection by all the three route resulted in CHIKV replication in the thigh muscles (Fig. 5A). CHIKV RNA copies were not detected in uninfected mice at 0, 2, 7 and 14 days p.i.. Mice inoculated with in i.v., i.m. and i.n. routes; CHIKV was not detected in the muscle tissues at 0 day p.i. (Fig. 5A). CHIKV appeared in the muscle tissues by 2 days p.i., persisted till 7 days p.i. and disappeared on 14 days p.i. (Fig. 5A). CHIKV inoculated via i.m. route could be detected in thigh muscle tissues at 2 h p.i. (753±101 CHIKV RNA copies). Therefore i.m. route was not preferred as it was difficult to distinguish newly replicated virus from the virus innoculum. Intra nasally inoculated mice exhibited the lower viral RNA copies in the thigh muscles (Fig. 5A). Since i.m. and i.n. routes were not yielded satisfactory results, therefore we used i.v. route for CHIKV infection.

Swiss albino and C57 BL/6 mice were infected by i.v. route and CHIKV RNA copies were measured in serum and muscle tissues from 1 day p.i. to 14 day p.i.. Infection of adult swiss albino and C57BL/6 mice with 1×10^6 PFU CHKV (100 µl of 10^7pfu/ml) CHIKV by i.v. route did not cause mortality. Clinical symptoms such as sluggishness and foot swelling were observed. A definite evidence of the replication of the virus was observed in muscle tissues (Fig. 5B). CHIKV RNA copies were detected in mice serum from 1 day p.i. till 9 days p.i. (Fig. 5 C & D). Viremia in i.v inoculated mice reached a peak by 3 days p.i., with viral loads ranging from 7×10^5 to 5×10^7 viral RNA copies/ml (Fig. 5 C & D).

siRNA inhibits the CHIKV replication in swiss albino mice

To assess whether siRNAs could protect mice from CHIKV infection, groups of CHIKV infected mice (1×10^6 PFU CHIKV; 100 µl of 10^7pfu/ml) were administered Chik-1 and Chik-5 siRNAs at 72 h p.i.. Swiss albino mice treated with E2 or ns1 siRNA with 250 µg per kg body weight (~6 µg/mice) showed ~3$\log_{10}$ inhibition, 500 µg per kg body weight (~12 µg/mice) showed 3$\log_{10}$ inhibition of CHIKV whereas at 1 mg per kg body weight (~25 µg/mice) siRNA led to 7$\log_{10}$ reduction in CHIKV copies (Fig. 6 A & B). Similar results were obtained in C57BL/6 mice (Fig. 6 C & D). We therefore administered 1 mg/kg body

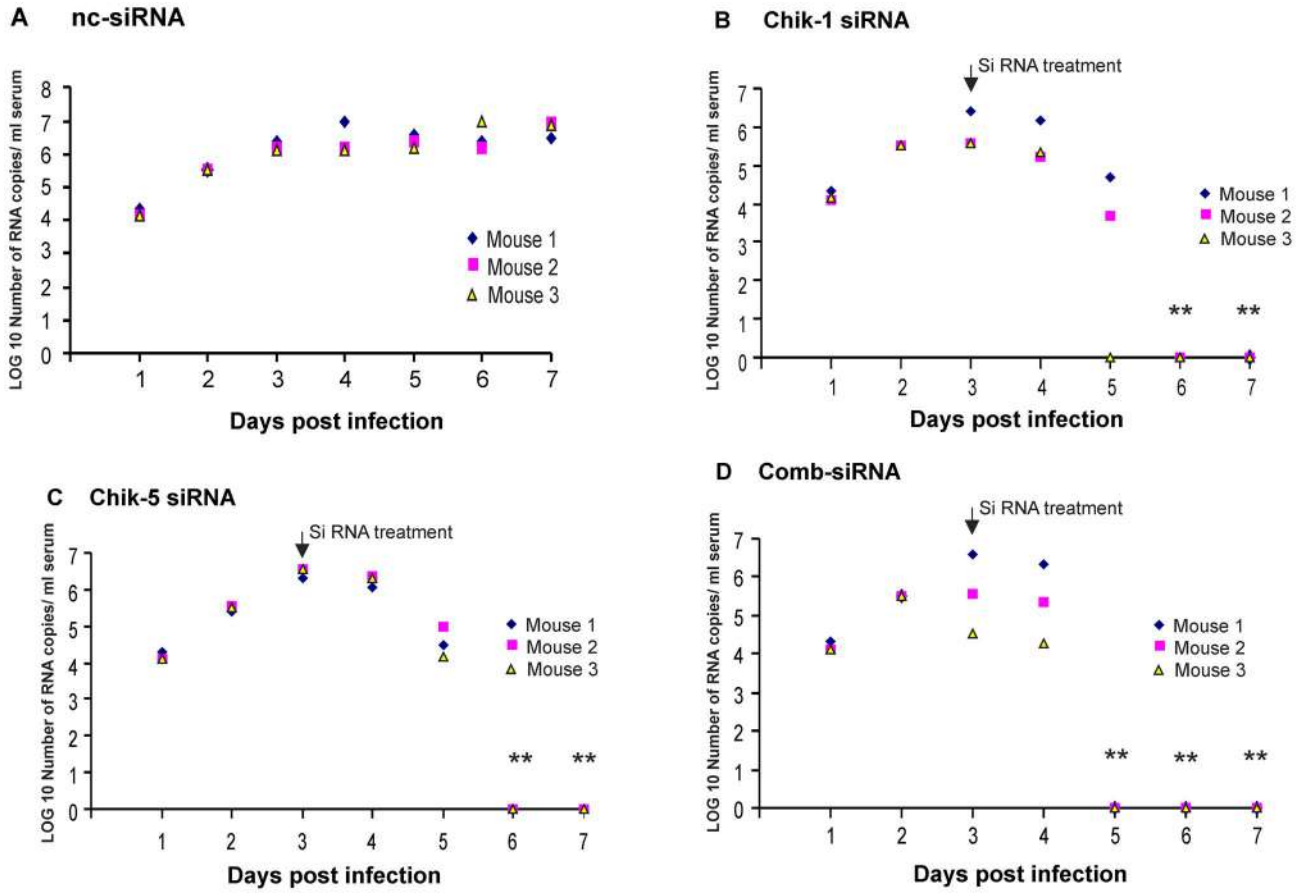

Figure 7. The reduction in CHIKV copies/ml serum after injection with siRNA Chik-1 and Chik-5 in Swiss albino mice infected with CHIKV. Swiss albino mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). After 72 h of p.i. mice were inoculated i.v. with **A**) ncsiRNA 1 mg/kg body weight **B**) 1 mg/kg body weight Chik-1 (n = 3), **C**) 1 mg/kg body weight Chik-5 (n = 3) and **D**) Comb-siRNA (n = 3) 1 mg/kg body weight each. At indicated time after injection of siRNA blood was collected from eye and RNA was isolated from serum. CHIKV E3 RNA was quantitated using real time RT-PCR. Values are given as LOG10 RNA copies/ml serum. Significance ANOVA, Dunnett's test: *$p<0.05$; **$p<0.01$.
doi:10.1371/journal.pntd.0002405.g007

weight (~25 µg/mice) siRNA in subsequent experiments. For all *in-vivo* experiments, Hiperfect reagent was used for delivery of siRNA. Chik-1, Chik-5 and Comb-siRNA administered at 72 h p. i. provided significant reduction in serum viral load as assessed by real time PCR (Fig. 7). At 48 h post siRNA injection, reduction with Chik-1 and Chik-5 was around 2.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) as compared to 0 h and ncsiRNA whereas 100% inhibition ($7\log_{10}$) was observed with Comb-siRNA (ANOVA Dunnet's test $p<0.01$). At 72 h p.i., administration of Chik-1, Chik-5 and Comb-siRNAs showed complete inhibition ($7\log_{10}$, ANOVA Dunnet's test $p<0.01$).

Inhibition of the CHIKV replication in C57BL/6 mice after treatment of siRNA

Chik-1, Chik-5, Comb-siRNA and ncsiRNA administered 72 h p.i. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) provided significant reduction in the serum viral load as assessed daily by real time PCR (Fig. 8). At 24 h and 48 h post siRNA treatment, 2.5 $\log_{10}$ and 3.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) reduction was recorded for all siRNAs, when compared to ncsiRNA. At 72 h post treatment, reduction with siRNA Chik-1, and Chik-5 was around 3.5 $\log_{10}$ (ANOVA Dunnet's test $p<0.05$) while Comb-siRNA showed 100% inhibition ($7\log_{10}$, ANOVA Dunnet's test $p<0.01$). Importantly, Comb-siRNA produced prolonged inhibitory effect when compared to individual siRNAs. In muscle tissues, CHIKV RNA reached peak by third day p.i., with viral loads ranging from 1×10^4 to 7×10^5 viral RNA copies/mg tissue (Fig. 8). At 24 h post-siRNA treatment ~2.5 $\log_{10}$ reduction in CHIKV RNA was noted with all the three siRNAs as compared to ncsiRNA control. At 72 h, all the siRNAs produced $4\log_{10}$ reduction in CHIKV RNA (100% inhibition, ANOVA Dunnet's test $p<0.01$). Similar results were seen when IFA was used to evaluate the effect of siRNA on CHIKV replication in muscle tissues that corroborated with real time PCR-based data (Fig. 9).

Histopathological evaluation of mice muscle tissues after CHIKV infection and siRNA treatment

Having demonstrated that Chik-1 and Chik-5 siRNA treatment significantly reduced the CHIKV titer in serum and muscle tissues, histopathology analysis was performed to determine the inflammation and infiltration in chikungunya infected and siRNAs treated tissues. Histopathological examination of CHIKV infected mice (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) showed pathological changes such as extensive necrosis, inflammation, pronounced monocyte/macrophage infiltrates and edema (Fig. 10). Such histopathological changes were prevented by systemic treatment either with Chik-1, Chik-5 individually or in Comb-siRNAs. At 3 days p.i., the muscle

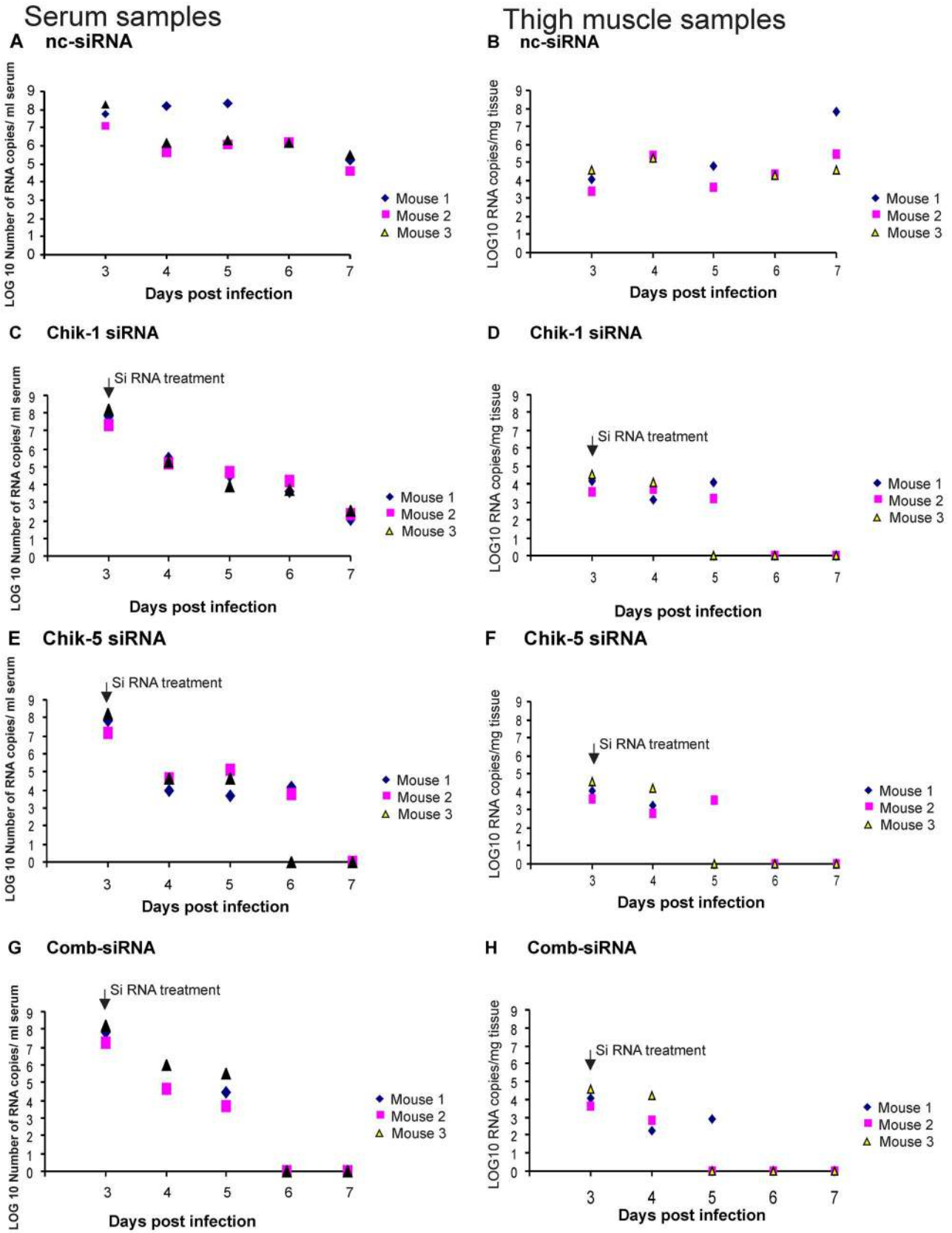

Figure 8. The reduction in CHIKV copies/ml serum after injection with siRNA Chik-1 and Chik-5 in C57BL/6 mice infected with CHIKV. C57BL/6 mice (n = 15) were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml) and viral RNA copies were checked in serum and muscle tissues. After 72 h p.i. mice were inoculated i.v. with 1 mg/kg body weight nc siRNA (**A** and **B**), 1 mg/kg body weight Chik-1 (n = 15) (**C** and **D**), 1 mg/kg body weight Chik-5 (n = 15) (E and F) and Comb-siRNA (n = 15) 1 mg/kg body weight each (**G** and **H**) and viral RNA copies were checked

in serum and muscle tissues at indicated time after injection. CHIKV E3 RNA was quantitated using real time RT-PCR. Values are given as LOG10 RNA copies/ml serum and LOG10 RNA copies/mg of tissue. Significance Dunnett's test: *$p<0.05$; **$p<0.01$.
doi:10.1371/journal.pntd.0002405.g008

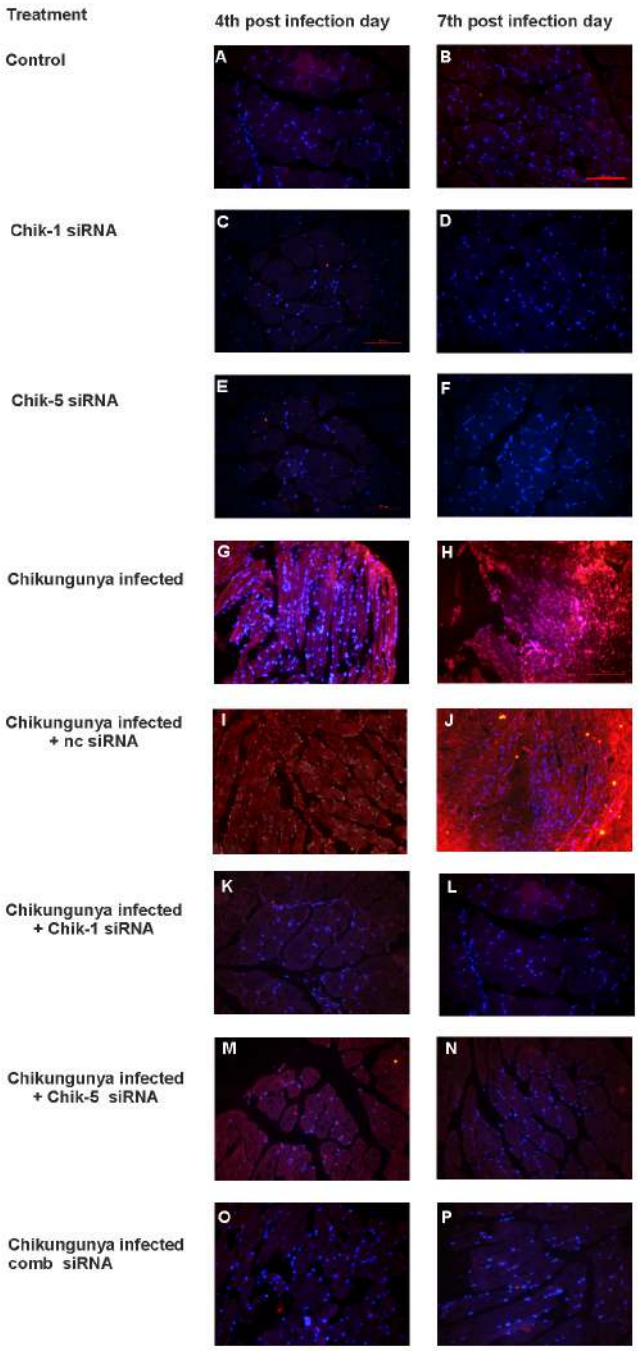

Figure 9. Detection of chikungunya in mouse muscle tissues using Immunofluorescence assay after chikungunya infection and siRNA treatment. C57BL/6 mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). PBS, ncsiRNA, E2 siRNA and ns1 siRNA injected mice showed absence of chikungunya antigen at 4th and 7th days p.i. (**A**, **B**, **C**, **D**, **E** and **F**). CHIKV infected muscles showed presence of chikungunya antigen (**G** and **H**), ncsiRNA treated CHIKV infected muscles showed presence of chikungunya antigen (**I** and **J**) whereas siRNA treated CHIKV infected mice muscle tissues showed the faint staining of chikungunya antigen (**K**, **L**, **M**, **N**, **O** and **P**) (Magnification ×200).
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tissues chikungunya infected mice showed mild inflammation of lymphocytes and monocytes. At 4 days p.i., chikungunya infected mice muscle tissues showed moderate inflammation of lymphocytes and monocytes, focal edema and focal necrosis whereas siRNA treated mice muscle tissues showed only mild inflammation. At 7 days p.i., extensive muscular necrosis with dense inflammation of lymphocytes and monocytes was observed in CHIKV infected and ncsiRNA treatment mice. On other hand, siRNA treatment preserved the morphological integrity of the muscle tissues with regeneration (Fig. 10). The muscle tissues from control mice infected with saline showed no pathological changes such as necrosis, edema, inflammation and infiltration of polymorphs (Fig. 10).

Expression levels of interferon genes after siRNA treatment

We tested if inhibition of CHIKV replication in mice was indeed sequence dependent and not because of non-specific antiviral interferon response. In the absence of CHIKV, Chik-1, Chik-5 and Comb-siRNA treatment did not significantly induced the α, β and γ interferon mRNA expression in hind limb muscle tissues (Table 3; Kruksal Wallis $p>0.05$). Similarly, siRNA treatment of CHIKV-infected mice did not show significant elevations in α, β and γ interferon gene expression in the hind limb muscle tissues (Table 3; Kruksal Wallis $p>0.05$). These results suggest that siRNA mediated reduction in CHIKV replication is sequence specific without any deleterious effect on the host.

Taken together, this first in vivo experiment clearly revealed that siRNA therapy is effective *in vivo* by reducing the clinical symptoms in the challenge-infected animals and was capable of significantly reducing virus replication in the serum and muscles.

Discussion

This study for the first time clearly shows the efficacy of ns1 and E2 siRNAs in combination, in inhibiting CHIKV replication in mice infected with the CHIKV (1×10^6 PFU CHIKV; 100 µl of 10^7pfu/ml, ANOVA Dunnet's test $p<0.01$). Importantly, a single i.v. inoculation of the siRNAs, 72 h after CHIKV infection could completely inhibit viral replication as evidenced by the absence of viral RNA in the muscles and serum. Though attractive, the therapeutic potential of siRNAs in treating viral diseases has been limited primarily because of the failure when evaluated in animal models and the absence of appropriate delivery systems. However, the success of *in-vivo* use of siRNAs in viral infection is noteworthy [17,23,24].

E2 and ns1 genes were chosen as the target genes because of the critical roles in viral replication. E2 and ns1 genes are highly conserved in CHIKV strains. CHIKV is expected to encode spikes on the virion surface, each formed by three E1–E2 heterodimers where the E1 glycoproteins mediate fusion and the E2 glycoproteins interact with the host receptor [26,27,28,30]. Together with nsP4, nsP1 is expected to catalyze the initiation of negative strand RNA synthesis. Nsp1 protein is also involved in methylation and capping of positive RNA [31]. Indeed, CHIKV nsp1, a 535 amino acid long protein contains consensus sequence at the N terminal region which is characteristic of Alphaviruses. Elimination of nsp1 abolishes the ability of CHIKV to replicate. We evaluated four different siRNAs each against ns1 and E2 genes when administered two h p.i. *in vitro*. Of these, Chik-1 (siRNA for E2 gene) and

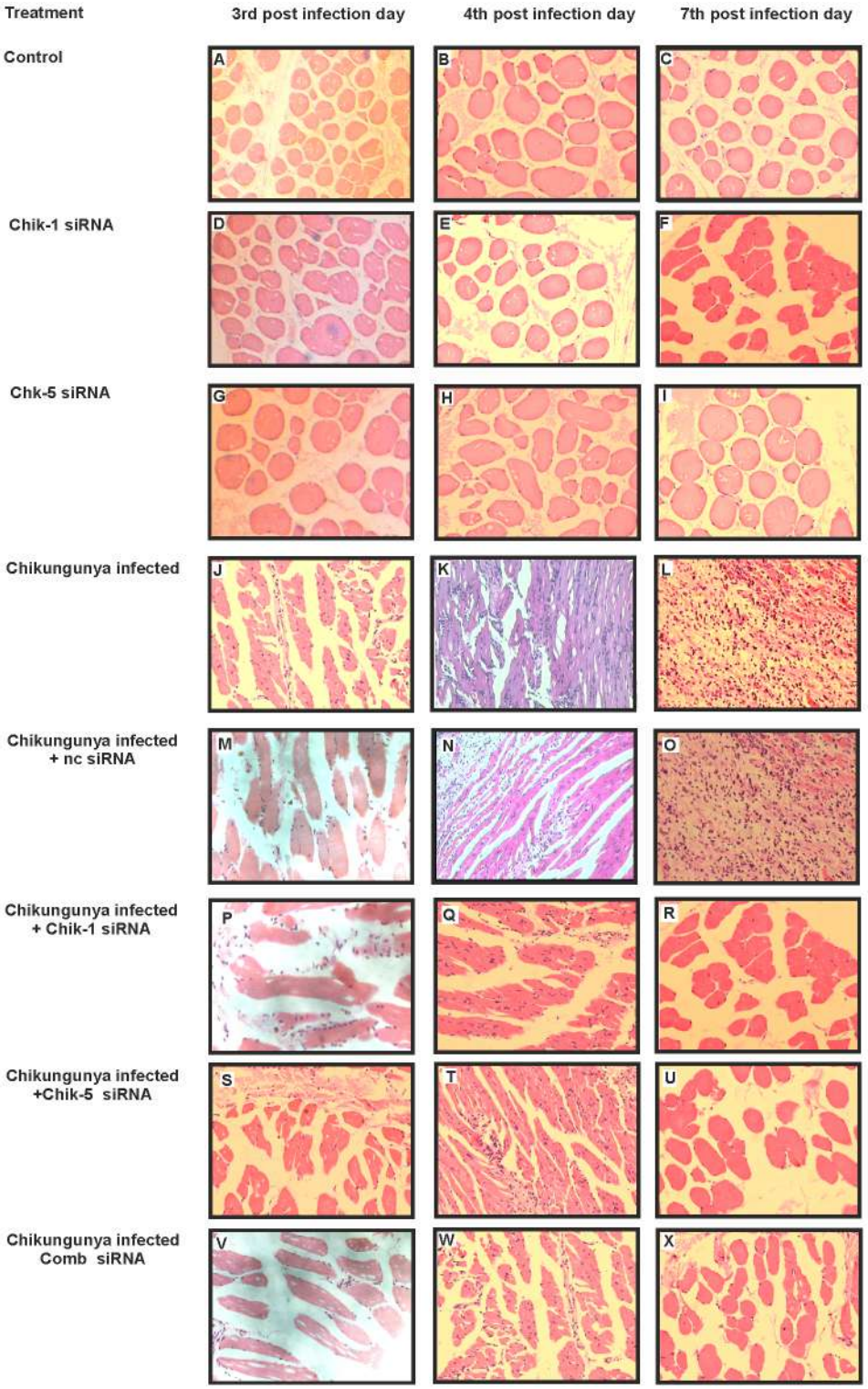

Figure 10. Histopathological changes in mouse muscle tissues after chikungunya infection and siRNA treatment. C57BL/6 mice were infected with CHIKV i.v. (1×10^6 PFU CHKV; 100 µl of 10^7pfu/ml). Hematoxylin/eosin-stained tissue sections were screened to investigate the pathological effects of siRNA treatment. PBS injected mice showed normal cellular organization (**A**, **B** and **C**). No significant cellular changes were observed in E2 siRNA treated mice (**D**, **E** and **F**) and ns1 siRNA treated mice (**G**, **H** and **I**). At 3 days p.i. mild inflammation and mild monocyte/macrophage infiltrates were observed (**J**, **M**, **P**, **S** and **V**). At 4 days p.i. and 7 days p.i. CHIKV infected and ncsiRNA treated muscles showed pronounced monocyte/macrophage infiltrates, necrosis and edema (**K**, **L**, N and **O**), whereas siRNA treated CHIKV infected mice muscle tissues showed the regeneration after treatment (**Q**, **R**, **T**, **U**, **W** and **X**) (Magnification ×400).
doi:10.1371/journal.pntd.0002405.g010

Table 3. Interferon response in hind limb muscle tissue of C57BL/6 mice after treatment with siRNAs.

Post infection period	Treatment			Relative fold change compared to control		
	Chikungunya infection	Chik-5 siRNA	Chik-1 siRNA	Interferon α	Interferon β	Interferon γ
				Mean (SD)	Mean (SD)	Mean (SD)
4 days p.i.						
(n = 3)	+			0.94 (0.88)	2.03 (1.31)	25.22 (21.12)
(n = 3)	+	+		2.83 (2.19)	1.81 (1.61)	8.25 (5.06)
(n = 3)	+		+	2.06 (1.77)	2.69 (2.21)	18.00 (16.80)
(n = 3)	+	+	+	1.21 (0.80)	3.99 (3.82)	4.55 (1.23)*
(n = 3)		+		1.54 (1.21)	5.18 (3.17)	1.09 (0.60)
(n = 3)			+	0.95 (0.66)	4.76 (3.23)	1.08 (0.64)
(n = 3)		+	+	2.19 (0.46)	6.00 (4.55)	7.09 (5.18)
5 days p.i.						
(n = 3)	+			0.11 (0.05)	0.41 (0.33)	371.73 (236.04)
(n = 3)	+	+		1.88 (1.48)	1.71 (1.54)	19.44 (11.93)
(n = 3)	+		+	4.65 (2.08)	4.74 (4.14)	44.38 (41.70)
(n = 3)	+	+	+	0.81 (0.30)	3.59 (3.49)	163.09 (65.36)*
(n = 3)		+		1.22 (0.53)	4.69 (1.06)	3.31 (1.91)
(n = 3)			+	0.60 (0.44)	6.54 (0.74)	0.97 (0.06)
(n = 3)		+	+	3.38 (2.63)	5.55 (4.75)	1.49 (2.00)
6 days p.i.						
(n = 3)	+			2.15 (2.05)	0.42 (0.27)	9.37 (5.87)
(n = 3)	+	+		2.50 (1.27)	3.61 (3.06)	6.06 (4.64)
(n = 3)	+		+	14.27 (9.47)	1.54 (1.35)	50.74 (42.74)
(n = 3)	+	+	+	5.15 (4.80)	4.85 (2.20)	344.23 (294.86)
(n = 3)		+		0.45 (0.10)	4.89 (3.59)	1.10 (0.74)
(n = 3)			+	0.62 (0.42)	5.71 (1.40)	0.68 (0.65)
(n = 3)		+	+	3.67 (3.47)	6.16 (5.59)	0.78 (0.64)
7 days p.i.						
(n = 3)	+			0.67 (0.56)	2.23 (1.90)	4071.74 (705.74)
(n = 3)	+	+		2.36 (1.98)	0.54 (0.05)	7.51 (1.50)
(n = 3)	+		+	18.10 (13.29)	6.44 (5.12)	2.15 (1.87)
(n = 3)	+	+	+	1.32 (1.31)	4.83 (0.33)	119.31 (91.42)
(n = 3)		+		0.58 (0.07)	2.67 (0.18)	1.13 (0.17)
(n = 3)			+	0.52 (0.02)	6.35 (3.68)	2.39 (0.28)
(n = 3)		+	+	2.17 (1.53)	5.74 (5.13)	27.38 (20.14)

Mice were treated with siRNA and gene expression changes in interferon alpha, beta and gamma was monitored in hind limb muscle tissues (n = 3 for each treatment) at 24 h, 48 h, 72 h and 96 h p.i.. Results expressed as $2^{-\Delta\Delta CT}$ were reported as mean ± standard deviation and were analyzed using Kruksal Wallis test.
*$p<0.05$ significantly different gene expression change as compared to chikungunya infected mice of respective time point.
doi:10.1371/journal.pntd.0002405.t003

Chik-5 (siRNA for ns1 gene) were most efficient in inhibiting CHIKV replication as assessed by multiple parameters such as real time PCR, plaque assay and IFA (Fig. 2, Fig. 3, Fig. 4, Table 2). The work described here utilizes targets against viral RNA sequences that are conserved, and invariant between different strains. The target sites of Chik-1 and Chik-5 siRNA are 100% conserved in all sequenced CHIKV genome. As compared to individual Chik-1 and Chik-5, the pool of Chik-1–4 and Chik5–8 siRNAs does not have any additive effect on the CHIKV inhibition. The combination therapy of Chik-1 and Chik-5 siRNAs provided additive effect and was found to be most effective as compared to individual siRNA in inhibiting CHIKV replication even at later time points. Thus, simultaneous silencing of multiple genes of CHIKV appeared to be better strategy for preventing viral replication. Recently Dash et al., [22] have used siRNA against ns3 and E1 genes of CHIKV and showed reduction in CHIKV replication by 65% by 48 h p.i. and not evaluated *in-vivo* [22]. *In vitro* studies employing Chik-1, Chik-5 and Comb-siRNAs showed more than 90% reduction in CHIKV replication with all three formulations, whereas complete inhibition of CHIKV replication was observed in *in vivo* studies. The observed efficient inhibition of CHIKV might be due to targeting of conserved sites, route of delivery or combination of both.

The next step was to evaluate these siRNAs in a suitable animal model. Recently C57BL/6 mice were established as susceptible murine models for CHIKV infection [32,33]. In the absence of an

immunocompetent mouse model replicating clinical symptoms in humans, we used Swiss albino and C57BL/6 adult mice for the evaluation of siRNAs. Though mice strains showed mild clinical symptoms (sluggishness and swelling of foot), replication of CHIKV was evident and the effect of siRNAs on CHIKV replication could be assessed employing several parameters (Fig. 5, Fig. 6, Fig. 7 and Fig. 8). SiRNAs were stable in Vero-E6 cells till 24 h post transfection (Fig. S1). We could not study the stability of siRNAs in mice because of low signal of cyanine 3 dye labeled siRNAs. However, it may be noted that previous studies have demonstrated uptake of siRNAs in the liver, kidney, lung endothelium and jejunum using standard i.v. injection of siRNA [34,35,36]. Despite the weaker siRNA uptake with standard intra venous administration, earlier reports suggest that this technique is still effective and may offer a potential route for systemic therapeutic use. Standard i.v. administration of siRNA efficiently delivered the siRNAs to various organs and resulted in efficient reduction of CHIKV titer.

Although chikungunya viremia has not been extensively studied in humans, studies on non human primates and mice suggest viremia of short duration, with viral loads ranging from 1×10^3 to 1.2×10^{10} viral RNA copies/ml [33,37,38,39,40]. It has been demonstrated that CHIKV can persist for longer time in animal models [37,40]. The findings of Morrison et al., [40] and Labadie et al., [37] indicate that though CHIKV is readily cleared from most tissues after the acute stage of infection, CHIKV RNA persists in joint tissues for at least 3–4 weeks after inoculation. In monkey model a clear relationship has been demonstrated between the inoculation dose and the period and magnitude of the viremia [37]. In current study we used the high dose of CHIKV (1×10^6 PFU) which might resulted in longer persistence of CHIKV RNA (7–8 days post inoculation). The results obtained in this study are consistent with findings of Morrison et al., [40] and Labadie et al., [37]. It has been demonstrated that CHIKV actively suppresses STAT activation by both type I and type II interferon [37,41]. In current study moderate suppression in Interferon α and Interferon β was observed in CHIKV infected mice. It is possible that CHIKV persistence observed in current study might be the combined effect of high dose of CHIKV, route of inoculation, active evasion of host innate or adaptive immune responses by the CHIKV. It will be interesting to evaluate the mechanisms involved in CHIKV persistence in C57BL/6 mice.

Similar to *in-vitro* studies, Comb-siRNA was more efficient than the individual components *in-vivo* studies also. Important point is that the siRNA was administered 48 or 72 h p.i. suggesting utility in CHIKV-infected hosts. CHIKV was detected in muscle tissues of infected mice inducing pathological changes such as severe necrosis and dense infiltration of monocytes and lymphocytes (Fig. 9 and 10). On the contrary, ~1 day after siRNA treatment, mild inflammation and infiltration of monocytes was observed while after ~4 days, regeneration and intact muscle morphology with no evidence of inflammation was recorded (Fig. 10). These results clearly demonstrate the therapeutic effect of siRNAs, especially Comb-siRNA, in virus-infected mice. Even a single dose administered 3 days p.i. could protect mice suggesting ability of the siRNAs in treating an established virus infection.

Under certain circumstances, siRNAs can induce the interferon (IFN) pathway and trigger inflammation [42,43,44]. It has been suggested that canonical small interfering RNA (siRNA) duplexes are potent activators of the mammalian innate immune system [42,43,44]. Synthetic siRNA in delivery vehicles that facilitate cellular uptake can induce high levels of inflammatory cytokines and interferons after systemic administration in mammals and in primary human blood cell cultures [42,43]. To differentiate the modes of protection offered by siRNAs, we determined expression levels of interferon α, β and γ interferon genes in the muscle tissues of different mice groups (Table 3). siRNAs alone did not induce significant induction of interferon genes; CHIKV-infected, siRNA treated mice did not show siRNA-induced interferon gene expression when compared to the virus infected mice. These results revealed that the observed inhibition of CHIKV replication was mainly because of characteristic activity of siRNAs.

In conclusion, Comb-siRNAs (E2 and ns1 genes) described by us provide an excellent therapeutic agent for chikungunya and should be further assessed in non-human primates. Need for a proper delivery system for use in humans remains an important issue.

Supporting Information

Figure S1 Stability of siRNAs. Cyanine 3 dye labelled Chik-1 and Chik-5 were transfected in Vero-E6 cells using Hiperfect reagent (Qiagen, Germany). After 4 h and 24 h cyanine 3 dye fluorescence signal was detected using fluorescence microscope (Nikon eclipse T2000S and Q capture pro 5.0 software).
(DOC)

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Author Contributions

Conceived and designed the experiments: DP MSP VAA. Performed the experiments: DP MSP SK MDG ABS SBS. Analyzed the data: MSP. Contributed reagents/materials/analysis tools: DP MSP SK MDG ABS SBS VAA. Wrote the paper: DP MSP VAA.

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