Ultrasound treatment inhibits SARS-CoV-2 in vitro infectivity

Shortened title: Viral load decrease with Ultrasound exposition therapy

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Abstract

Background

COVID-19 (coronavirus disease 2019) is a disease caused by infection with the severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2), affecting millions of people worldwide, with a high rate of deaths. The present study aims to evaluate ultrasound (US) as a physical method for virus inactivation.

Materials and methods

The US-transductor was exposed to the SARS-CoV-2 viral solution for 30 minutes. Vero-E6 cells were infected with medium exposure or not with the US, using 3-12, 5-10, or 6-18MHz as frequencies applied. We performed confocal microscopy to determine virus infection and replicative process. Moreover, we detected the virus particles with a titration assay.

Results

We observed an effective infection of SARS-CoV-2 Wuhan, Delta, and Gamma strains in comparison with mock, an uninfected experimental group. The US treatment was able to inhibit the Wuhan strain in all applied frequencies. Interestingly, 3-12 and 6-18MHz did not inhibit SARS-CoV-2 delta and gamma variants infection, on the other hand, 5-10MHz was able to abrogate infection and replication in all experimental conditions.

Conclusions

These results show that SARS-CoV-2 is susceptible to US exposure at a specific frequency 5-10MHz and could be a novel tool for reducing the incidence of SARS-CoV-2 infection.

Keywords: Ultrasound, SARS-CoV-2, virucidal effect, COVID-19

Main text

Introduction

Critical situations and great challenges facing humanity historically tend to drive scientific advances. It was no different in the current pandemic. Since 2020, a large mobilization of scientists and public and private scientific entities has been observed, seeking to better understand the viruses and diseases caused by their infection in humans, as well as the solutions to the crisis, whether through treatment or vaccines, or even tests and sensors. Many works in different areas of science were proposed in areas as distant as biology, physics, medicine, engineering, computing, and others areas, focusing on solutions to face the problem.

Among different works, one of them caught our attention. Wierzbicki et al, in 2021, proposed the possibility of acoustic waves at the Ultrasound (US) frequency being able to damage and consequently neutralize the SARS-CoV-2 virus. The authors found high frequencies, between 100 and 500 MHz as possible resonance points of the virus carapace and its t-spike proteins. In a second work, Wierzbicki and Bai, in 2022, carried out a new theoretical study suggesting that frequencies, lower between 1 and 20 MHz, can also damage the SARS-CoV-2 spikes structures.

In this work, we carried out experiments to verify if the SARS-CoV-2 virus can be inactivated by resonance caused by sound waves at the US frequency. Although both theoretical works mention the physical possibility of ultrasound harmonics interacting with SARS-CoV-2 spike proteins, this has not yet been experimentally proven. In this work, in vitro experiments are carried out, the results of which validate previous theoretical works and strongly suggest that ultrasound can be used to neutralize SARS-CoV-2.

Materials and methods

Virus stock production

The SARS-CoV-2 parental Wuhan, SARS CoV-2 gamma (P1), and SARS CoV-2 delta variants were used for *in vitro* experiments, under strict biosafety level 3 (BSL3) conditions at the Ribeirao Preto Medical school (Ribeirao Preto, Brazil).

Briefly, viral inoculum (1:100 ratio) was added to the Vero E6 cells, and the culture was incubated (48 h, 37 °C, 5% CO_2 humidified atmosphere) in DMEM without FBS but supplemented with antibiotic/antimycotic mix (Penicillin 10,000 U/mL; Streptomycin 10,000 µg/mL; Sigma-Aldrich; cat. P4333) to optimize virus adsorption to the cells. After confirming the cytopathic effects of the viral replication over cell monolayer, cells were detached by scraping, harvested, and centrifuged (10000 ×g, 10 minutes, room temperature). The resulting supernatants were stored at -80 °C until use. SARS CoV-2 variants titration was assessed using standard limiting dilution to determine the 50% tissue culture infectious dose (TCID50).

In vitro SARS-CoV-2 infection and US-exposure

Vero E6 cells were infected with SARS-CoV-2 before being exposed to 3-12, 5-10, or 6-18 MHz US frequencies from linear array transducers at room temperature for 30 minutes. An ultrasound high-resolution machine for routine images, MyLab 60 (Esaote) or Envisor (Philips), was used. Cells were infected at a multiplicity of infection (MOI) of 1.0 with infectious clone SARS-CoV-2 or mock with infection media for 24 hours to evaluate the infection and replication process by immunofluorescence and confocal microscopy. The productive viral particle was assessed by TCID50 assay. The treatment was performed in technical triplicate. The culture medium temperature was measured as a control using a thermal camera (FLIR One Pro, Flir).

Immunostaining and confocal

For SARS-CoV-2 detection *in vitro*, Vero-E6 cells were plated in 24-well plates containing glass coverslips, fixed with PFA 4% at RT for 10 minutes, and blocked with 1% bovine serum albumin (BSA; Sigma-Aldrich; cat. A7906) and 22.52 mg/mL glycine (Sigma-Aldrich; cat. G8898) in PBST (Phosphate Buffer Saline + 0.1% Tween 20) at RT for 2 hours. The coverslips were stained with the following antibodies: rabbit anti-spike protein (Invitrogen; cat. 703959; 1:500) and mouse anti-dsRNA (J2; dsRNA, SCICONS English & Scientific Consulting Kft., clone J2-1909, cat.10010200; 1:1,000). After this, samples were washed in PBS and incubated with secondary antibodies: alpaca anti-mouse IgG AlexaFluor 488

(Jackson ImmunoReseacher; Cat. 615-545-214; 1:1,000) and alpaca anti-rabbit IgG AlexaFluor 594 (Jackson ImmunoReseacher; Cat. 611-585-215; 1:1,000). Slides were then mounted using Vectashield Antifade Mounting Medium with DAPI (Vector Laboratories; cat. H-1200-10). Images were acquired by Axio Observer combined with an LSM 780 confocal microscope (Carl Zeiss) at 630X magnification at the same setup of zoomed and laser rate Images were acquired and analyzed using Fiji by Image J.

Titration TCID50

To evaluate the effect of exposure to the US on SARS-CoV-2 infectivity, the virus stock was diluted 1:100 in each of the following: DMEM and/or US-exposed SARS-CoV-2. These two SARS-CoV-2 preparations were incubated for 1 min at room temperature, serially diluted 10-fold in DMEM, and then 100 µL of each dilution was inoculated in quadruplicate monolayers to determine the virus titer by TCID50 in Vero CCL-81 cells in 96-well plates.

Statistics

Statistical significance was determined by one-way ANOVA followed by Bonferroni's post hoc test. $P<0.05$ was considered statistically significant. Statistical analyses and graph plots were performed and built with GraphPad Prism 9.3.1 software.

Results

The potential virucidal effects of US on SARS-CoV-2 were experimentally assessed for different frequencies and SARS-CoV-2 virus strains, such as delta and gamma variants. We exposed a solution containing SARS-CoV-2 particles with US-transductor for 30 minutes (**Figure 1A**). Then, we infected Vero-E6 cells with culture medium exposed or not with the US, using 3-12, 5-10, or 6-18MHz as frequencies applied. We performed immunofluorescence and confocal microscopy 24 hours post-infection to determine virus infection with staining for SARS-CoV-2 spike protein and double-stranded(ds) RNA (dsRNA), which

indicates a replicative process. The US treatment was able to inhibit the Wuhan strain in 3-12, 5-10, and 6-18 MHz frequencies. The virucidal effect in delta or gamma variants was observed only in the 5-12MHz group. We did not observe a virucidal effect in 6-18MHz (**Figure 1B**).

We next investigated whether the US exposition in SARS-CoV-2 can affect the number of productive SARS-CoV-2 particles. We observed an effective infection of SARS-CoV-2 Wuhan, delta, and gamma strains in comparison with mock, an uninfected experimental group (**Figure 2**). In the Wuhan group, we observed the reduction of viral tilter at 3-12 and 5-10MHz (**Figure 2A**). The 6-18MHz frequency did not inhibit the SARS-CoV-2 viral tilter (**Figure 2**). Interestingly, the 3-12MHz frequency did not reduce SARS-CoV-2 delta and gamma strains. Using aesthetic ultrasound with 1-3 MHz, we did not observe an effect in neutralizing SARS-CoV-2 (Data not shown). In addition, the temperature of the culture medium did not alter upon US exposition (**Supplementary Figure 1**). These results show that SARS-CoV-2 is susceptible to US exposure at a specific frequency 5-10MHz and could be a novel tool for reducing the incidence of SARS-CoV-2 infection.

Discussion

The development of effective virus inactivation methods is of great importance to control their SARS-CoV-2 spread(Patterson et al., 2020; Rabenau et al., 2005; Darnell et al., 2004). This study investigated the effect of low-intensity US on the infectivity SARS-CoV-2 virus.

Wierzbicki et al, in 2021, proposed the possibility of acoustic waves at the US frequency being able to damage and consequently neutralize the SARS-CoV-2 virus (Wierzbicki et al., 2021). The study carried out was theoretical. The authors used finite element modeling and simulated the vibration interaction caused by ultrasound resonance with the virus. The work did not consider the propagation medium, and the authors found high frequencies between 100 and 500 MHz as possible resonance points of the virus carapace and its t-spike proteins. In a second work, Wierzbicki and Bai, in 2022, carried out a new theoretical study suggesting that frequencies, lower between 1 and 20 MHz, can also damage the α-helices and tropocollagen molecules of the SARS-CoV-2 spikes structures, consequently neutralizing the virus (Wierzbicki and Bai, 2022).

Frequencies of this magnitude would allow the use of US equipment for everyday use in medicine, properly regulated and safe for human use, in neutralizing SARS-CoV-2. Indeed, using US devices from medical diagnostics, we experimentally validate that lower frequencies can inhibit the infectivity of SARS-CoV-2. Interestingly, our results indicate a specific frequency rate of US exposition in an aqueous culture medium. We showed that 5-10 MHz was the most effective in reducing the SARS-CoV-2 viable particles, including the SARS-CoV-2 strains, gamma, and delta, compared with other used frequencies. Of note, Soto-Torres et al, in 2021 showed no significant differences in abnormal fetal US and Doppler findings observed between pregnant women who were positive for SARS-CoV-2 and controls that indicated equipment safety in humans (Soto-Torres et al., 2021). The increase in temperature is related to the US exposition and elevated temperature inhibits SARS-CoV-2 replication (Ghoshal et al., 2011; Herder et al., 2021). We did not observe differences in the temperature of the culture medium during the US exposition. This result supports the specific virucidal effect of US treatment.

Further testing, using US-exposition to determine the microscopy-affected virus structure and different time points may help clarify the mechanisms involved, develop the optimal time for inactivation of SARS-CoV-2, and perform in vivo experiments with preclinical models.

Conclusion

It was clearly shown that lower frequencies of the US contribute to SARS-CoV-2 virus inactivation. In addition, influences on virus inactivation occurred in different applied energy ranges without the interference of temperature. In addition, this novel method could potentially be combined with existing physical, and chemical methods and antiviral agents.

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Figure legends

Figure 1 – US treatment inhibits SARS-CoV-2 infection and replication

(A) Representative model of US exposition. **(B)** Immunofluorescence analysis of Spike (green) and dsRNA (red) expression of SARS-CoV-2-infected Vero-E6 cells and treated with US. DAPI (blue) was used for nuclei staining. Scale bar indicates 50 µm. Data are representative of at least two independent experiments.

Figure 2 – US treatment reduces infectious SARS-CoV-2

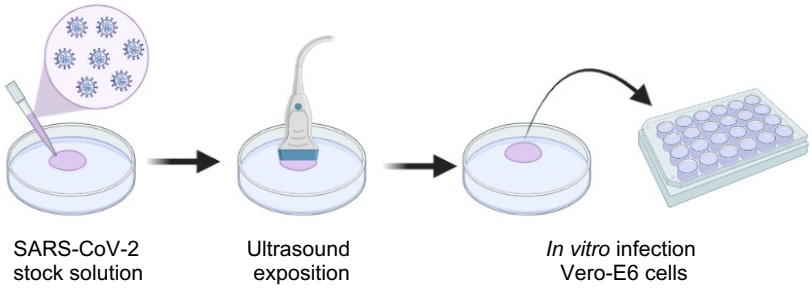
Vero-E6 cells were treated with a US-treated medium for 30 min. Titration of infectious SARS-CoV-2 Wuhan strain (**A**), Delta strain (**B**) and Gamma (**C**) by TCID50 assay Data are representative of at least two independent experiments and are shown as mean ± SEM. P values were determined by one-way ANOVA Followed by Bonferroni's post hoc test.

Supplementary Figure 1 – US treatment did not alter medium culture temperature

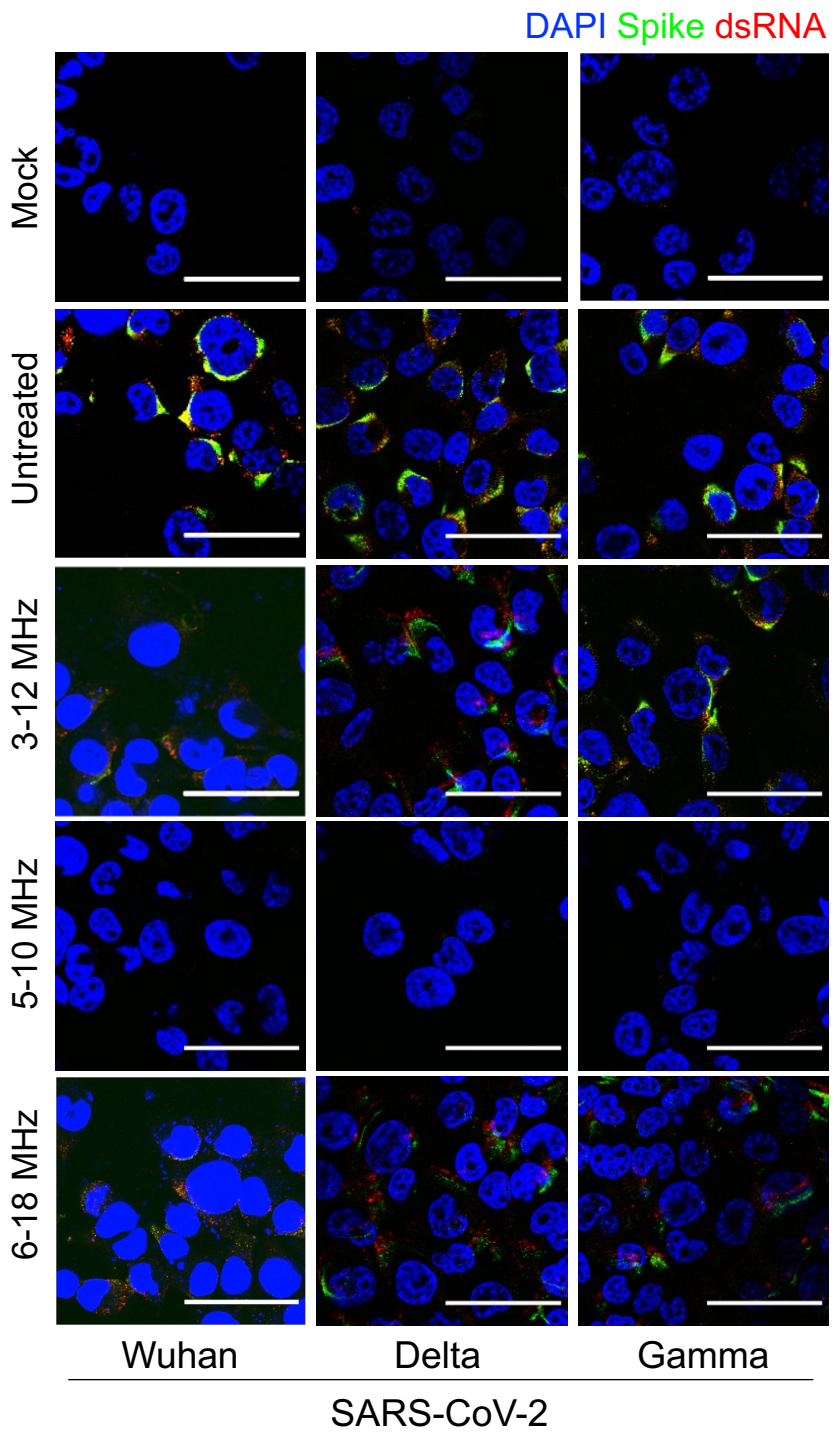
Quantification of DMEM medium culture temperature by a thermal camera for 30 min post US exposition upon different frequencies.

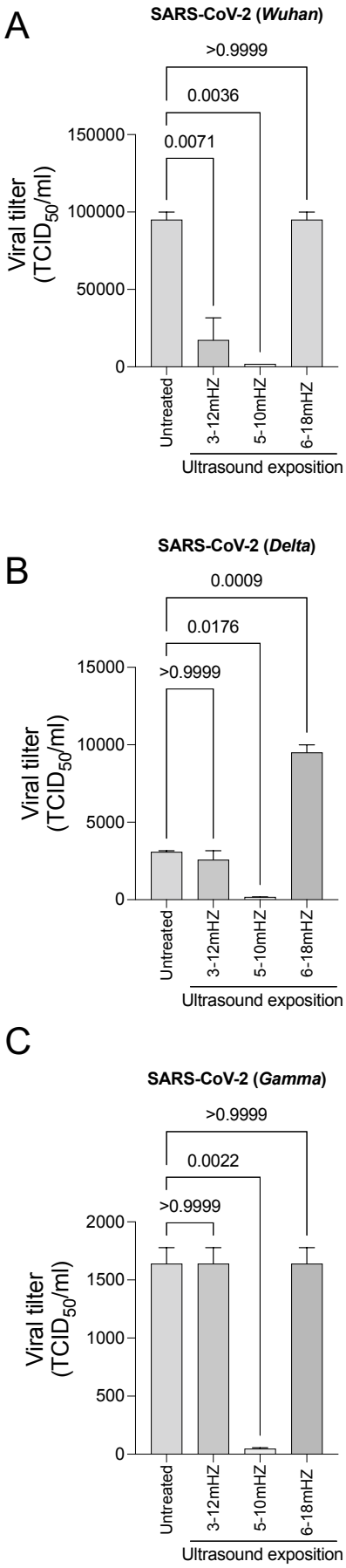
Figure 1

A


SARS-CoV-2
stock solution
Ultrasound
exposition
In vitro infection
Vero-E6 cells

B


DAPI Spike dsRNA
Mock
Untreated
3-12 MHz
5-10 MHz
6-18 MHz
Wuhan
Delta
Gamma
SARS-CoV-2


A
SARS-CoV-2 (Wuhan)
>0.9999
0.0036
0.0071
150000
100000
50000
0
Viral tilter (TCID50/ml)
Untreated
3-12mHZ
5-10mHZ
6-18mHZ
Ultrasound exposition
B
SARS-CoV-2 (Delta)
0.0009
0.0176
>0.9999
15000
10000
5000
0
Viral tilter (TCID50/ml)
Untreated
3-12mHZ
5-10mHZ
6-18mHZ
Ultrasound exposition
C
SARS-CoV-2 (Gamma)
>0.9999
0.0022
>0.9999
2000
1500
1000
500
0
Viral tilter (TCID50/ml)
Untreated
3-12mHZ
5-10mHZ
6-18mHZ
Ultrasound exposition

Supplementary Figure 1