

1 **Ultrasound treatment inhibits SARS-CoV-2 in vitro infectivity**

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3 **Shortened title:** Viral load decrease with Ultrasound exposition therapy

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1 **Abstract**

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3 **Background**

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

8

9 **Materials and methods**

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

15

16 **Results**

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

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24 **Conclusions**

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

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

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1 **Main text**

2 **Introduction**

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

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

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

28

29 **Materials and methods**

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31 *Virus stock production*

32 The SARS-CoV-2 parental Wuhan, SARS CoV-2 gamma (P1), and SARS CoV-
33 2 delta variants were used for *in vitro* experiments, under strict biosafety level 3
34 (BSL3) conditions at the Ribeirao Preto Medical school (Ribeirao Preto, Brazil).

1 Briefly, viral inoculum (1:100 ratio) was added to the Vero E6 cells, and the culture
2 was incubated (48 h, 37 °C, 5% CO₂ humidified atmosphere) in DMEM without
3 FBS but supplemented with antibiotic/antimycotic mix (Penicillin 10,000 U/mL;
4 Streptomycin 10,000 µg/mL; Sigma-Aldrich; cat. P4333) to optimize virus
5 adsorption to the cells. After confirming the cytopathic effects of the viral
6 replication over cell monolayer, cells were detached by scraping, harvested, and
7 centrifuged (10000 ×g, 10 minutes, room temperature). The resulting
8 supernatants were stored at -80 °C until use. SARS CoV-2 variants titration was
9 assessed using standard limiting dilution to determine the 50% tissue culture
10 infectious dose (TCID₅₀).

11

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

13 Vero E6 cells were infected with SARS-CoV-2 before being exposed to 3-12, 5-
14 10, or 6-18 MHz US frequencies from linear array transducers at room
15 temperature for 30 minutes. An ultrasound high-resolution machine for routine
16 images, MyLab 60 (Esaote) or Envisor (Philips), was used. Cells were infected at
17 a multiplicity of infection (MOI) of 1.0 with infectious clone SARS-CoV-2 or mock
18 with infection media for 24 hours to evaluate the infection and replication process
19 by immunofluorescence and confocal microscopy. The productive viral particle
20 was assessed by TCID₅₀ assay. The treatment was performed in technical
21 triplicate. The culture medium temperature was measured as a control using a
22 thermal camera (FLIR One Pro, Flir).

23

24 *Immunostaining and confocal*

25 For SARS-CoV-2 detection *in vitro*, Vero-E6 cells were plated in 24-well plates
26 containing glass coverslips, fixed with PFA 4% at RT for 10 minutes, and blocked
27 with 1% bovine serum albumin (BSA; Sigma-Aldrich; cat. A7906) and 22.52
28 mg/mL glycine (Sigma-Aldrich; cat. G8898) in PBST (Phosphate Buffer Saline +
29 0.1% Tween 20) at RT for 2 hours. The coverslips were stained with the following
30 antibodies: rabbit anti-spike protein (Invitrogen; cat. 703959; 1:500) and mouse
31 anti-dsRNA (J2; dsRNA, SCICONS English & Scientific Consulting Kft., clone J2-
32 1909, cat.10010200; 1:1,000). After this, samples were washed in PBS and
33 incubated with secondary antibodies: alpaca anti-mouse IgG AlexaFluor 488

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

8

9 *Titration TCID50*

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

16

17 *Statistics*

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

22

23 **Results**

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

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

5 We next investigated whether the US exposition in SARS-CoV-2 can affect the
6 number of productive SARS-CoV-2 particles. We observed an effective infection
7 of SARS-CoV-2 Wuhan, delta, and gamma strains in comparison with mock, an
8 uninfected experimental group (**Figure 2**). In the Wuhan group, we observed the
9 reduction of viral titer at 3-12 and 5-10MHz (**Figure 2A**). The 6-18MHz frequency
10 did not inhibit the SARS-CoV-2 viral titer (**Figure 2**). Interestingly, the 3-12MHz
11 frequency did not reduce SARS-CoV-2 delta and gamma strains. Using aesthetic
12 ultrasound with 1-3 MHz, we did not observe an effect in neutralizing SARS-CoV-
13 2 (Data not shown). In addition, the temperature of the culture medium did not
14 alter upon US exposition (**Supplementary Figure 1**). These results show that
15 SARS-CoV-2 is susceptible to US exposure at a specific frequency 5-10MHz and
16 could be a novel tool for reducing the incidence of SARS-CoV-2 infection.

17

18 **Discussion**

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

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

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

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

21

22 **Conclusion**

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

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1 **Figure legends**

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

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

9

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

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

16

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

19 Quantification of DMEM medium culture temperature by a thermal camera for
20 30 min post US exposition upon different frequencies.





