

Iota-carrageenan prevents the replication of SARS-CoV-2 on an in vitro respiratory epithelium model

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12 Keywords: SARS-CoV-2₁, COVID-19₂, iota-carrageenan₃, respiratory epithelium₄, antivirals₅.

13 **Abstract**

14 There are, except for remdesivir, no approved antivirals for the treatment or prevention of SARS-
15 CoV-2 infections. Iota-carrageenan formulated into a nasal spray has already been proven safe and
16 effective in viral respiratory infections. We explored this antiviral activity in Calu-3, a human
17 respiratory model cell line. A formula of iota-carrageenan and sodium chloride, as a nasal spray,
18 already approved for human use, effectively inhibited SARS-CoV-2 infection in vitro, providing a
19 more substantial reference for further clinical studies or developments.

20 **1 Introduction**

21 The severe acute respiratory coronavirus 2 (SARS-CoV-2) is responsible for the currently ongoing
22 pandemic coronavirus disease (COVID-19), counting more than 144.878.978 confirmed cases and
23 more than 3.075.042 deaths worldwide by April 23, 2021 (Dong et al., 2020). There are still no
24 adequate therapeutic or preventive medicines for COVID-19; repurposing established medications
25 with recognized safety profiles is a possible approach for preventing or treating the disease and
26 shortening the time-consuming drug development stages.

27 During the first days of the infection, the virus replicates mainly in the nasal cavity and the
28 nasopharynx; therefore, nasal sprays with antiviral activity would reduce the viral load in these
29 cavities.

30 Marine-derived polysaccharides, such as carrageenans, are a family of linear sulfated polysaccharides
31 extracted from red seaweeds, widely used as thickening agents and stabilizers for food. Besides these
32 properties, the iota-carrageenan demonstrated antiviral activity against several viruses, including
33 respiratory viruses such as human rhinovirus, influenza A H1N1, and common cold coronavirus
34 (Grassauer et al., 2008; Leibbrandt et al., 2010; Morokutti-Kurz et al., 2015). Iota-carrageenan
35 inhibits virus infection mainly based on its interaction with the surface of viral particles, preventing

36 them from entering cells and also trapping the viral particles released from the infected cells. It has
37 also been shown that their inhibitory activity also relies on affecting the viral replication cycle at
38 different steps, like entry and genome replication, and additionally activates the host's antiviral
39 immune response (Gomaa and Elshoubaky, 2016; Chen et al., 2020; Hans et al., 2020).

40 Iota-carrageenan formulated into a nasal spray has already been proven safe and effective in the
41 common cold treatment (Koenighofer et al., 2014). Based on these observations, the hypothesis has
42 been raised that a nasal spray with iota-carrageenan could be effective against SARS-CoV-2. It has
43 recently been described that iota-carrageenan has activity against the SARS-CoV-2 virus and its
44 Spike Pseudotyped Lentivirus (SSPL) in Vero E6 cell culture (Bansal et al., 2020; Morokutti-Kurz et
45 al., 2020; Song et al., 2020). The Vero E6 cell line, originally derived from African green monkey
46 kidney, is deficient for interferon- α (IFN α) and - β (IFN β) due to genetic deletions, for instance highly
47 susceptible to a vast number of different viruses, like measles virus, rubella virus, arboviruses,
48 adenoviruses, influenza, and some coronavirus, including SARS-CoV-2 (Osada et al., 2014; Barrett
49 et al., 2017; Banerjee et al., 2020).

50 Various studies have proposed the need to study SARS-CoV-2 infection in human respiratory
51 epithelium, in order to get closer to the central target tissue of the disease in patients (Holwerda et al.,
52 2020). Calu-3 is a non-small-cell lung cancer cell line that grows in adherent culture and displays
53 epithelial morphology. This cell line is considered a sensitive and efficient preclinical model to study
54 human respiratory processes and diseases (Zhu et al., 2010). Upon stimulation with viruses or
55 environmental toxins, the Calu-3 cell line synthesizes and releases different cytokines, including IL-6
56 (Zhu et al., 2008), which play a central role in the inflammatory cascade associated with more severe
57 COVID-19 (Gubernatorova et al., 2020).

58 The kinetics of SARS-CoV-2 infection show differences between Vero E6 and Calu-3 cells, most
59 probably related to the differential expression of the angiotensin-converting enzyme 2 (ACE2) entry
60 receptor and other facilitating molecules like the cellular serine protease TMPRSS2. Calu-3 cells
61 express ACE2 on the apical membrane domain and are infected by SARS-CoV-2 via this route. The
62 higher number of Vero E6 infected cells is also associated with the differences in viral entry pathway
63 and the expression of pro-apoptotic proteins, which increased drastically in these cells but not in
64 Calu-3 cells (Banerjee et al., 2020; Murgolo et al., 2021; Park et al., 2021; Saccon et al., 2021).

65 In this study, we assessed the effect of carrageenan as a viral infection inhibitor in an in vitro
66 respiratory epithelium model, providing a set of data that support previous results and that it could be
67 helpful against SARS-CoV-2 infection when applied in a formulation as a nasal spray.

68 **2 Materials and Methods**

69 **2.1 Cells and Virus**

70 African green monkey kidney Vero E6 cells (ATCC[®] CRL-1586[™]) and human airway epithelial
71 Calu-3 cells (ATCC[®] HTB-55[™]) were obtained from the American Type Culture Collection. The
72 Calu-3 cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Corning, NY, USA)
73 containing 10% fetal bovine serum (FBS, Thermo Fisher Scientific, Waltham, MA, USA), 100 U/ml
74 penicillin, and 100 μ g/ml streptomycin (Thermo Fisher Scientific, Waltham, MA, USA). The Vero
75 E6 cells were cultured in complete minimal essential medium (c-MEM) (Corning, NY, USA),
76 supplemented with 5% FBS (Thermo Fisher Scientific, Waltham, MA, USA). The cells were
77 incubated in 95% air and 5% CO₂ at 37°C.

78 The SARS-CoV-2 isolate was kindly provided by Dr. Sandra Gallegos (National University of
79 Córdoba, Argentina). Viral master seed stock was prepared using T175 flasks of Vero E6 cells. Each
80 flask was harvested on day two post-infection, and the supernatant was centrifuged twice at 220 x g
81 for 15 minutes to remove cellular debris. The titer of virus stock was determined by plaque assay on
82 Vero E6 cells and expressed as plaque-forming units per ml (pfu/ml). The experiments using the
83 virus were carried out in BSL3 facilities from the School of Medicine at the University of Buenos
84 Aires.

85 **2.2 Preparation of sample formulations**

86 Solutions with iota-carrageenan and sodium chloride were prepared using a sterile nasal spray for
87 therapeutic use. All the formulations and placebos were prepared at Laboratorio Pablo Cassará S.R.L.
88 (Argentina) under aseptic conditions. The composition of active and placebo formulations is depicted
89 in Table 1.

90 To determine antiviral efficacy of formulations by titer reduction assay, sample formulations were
91 used at a final iota-carrageenan concentration of 600 µg/ml; 60 µg/ml, 6 µg/ml, 0.6 µg/ml and 0,06
92 µg/ml. An equivalent concentration of placebos was used for titer reduction assay as controls.

93 **2.3 Viability cellular assays**

94 Calu-3 cells were seeded in 96-well tissue culture microplates at 3×10^4 cells/well, and incubated
95 overnight at 37°C under 5% CO₂. Then, the cells were treated or not with iota-carrageenan 600, 60,
96 6, 0.6, and 0.06 µg/ml or vehicle in culture medium for 48 h at 37°C. After incubation, cells were
97 washed and treated with MTS/PMS (CellTiter 96® Aqueous Non-Radioactive Cell Proliferation
98 Assay, Promega, USA).

99 **2.4 Infection assays**

100 In three independent experiments, Calu-3 cells were seeded in 96-well tissue culture microplates at
101 3×10^4 cells/well. After 48 h of incubation at 37°C, treated or not with iota-carrageenan 600, 60, 6,
102 0.6, and 0.06 µg/ml or vehicle and 2 h later infected with SARS-CoV-2 (multiplicity of infection
103 (MOI) = 0.01 and 0.1) in serum-free DMEM (Thermo Fisher) for 1 h at 37°C. Then, cells were
104 washed and placed in culture medium for 48 h. After that, supernatants were harvested and stored at -
105 80°C.

106 **2.5 Viral titration**

107 Vero E6 cells were seeded into 96-well microplates and grown overnight at 37°C under 5% CO₂.
108 Tenfold dilutions of virus samples from Calu-3 cells were added to monolayers of 80% confluent
109 Vero cells at 37°C for 1 h. After incubation, the inoculum was removed, and monolayers were
110 overlaid with DMEM. The cells were incubated at 37°C for 72 h and fixed using 4% formaldehyde.
111 Finally, cells were stained with 0.1% crystal violet in 20% ethanol and counted. Virus endpoint titer
112 was determined using the Reed-Muench formula and expressed as 50% tissue culture infectious dose
113 (TCID₅₀) per ml.

114 **2.6 Statistical analysis**

115 In the cellular viability assays, One-way ANOVA followed by Dunnett's multiple comparisons test
116 was performed, and in the infection assays, the results were analyzed statistically by two-way

117 ANOVA, both using GraphPad Prism version 9.1.0, GraphPad Software, San Diego, California USA,
118 www.graphpad.com.

119 **3 Results**

120 The antiviral effects of iota-carrageenan on SARS-CoV-2 were tested in a dose-dependent manner. In
121 the first set of experiments, Vero E6 cells were pre-treated with different iota-carrageenan
122 concentrations (600 µg/ml to 0.06 µg/ml), and cell viability was quantified (Figure 1A). No
123 difference in cell viability was observed in iota-carrageenan treated cells compared to vehicle-treated
124 control cells.

125 Next, Vero cells were pre-treated with iota-carrageenan (600 µg/ml to 0.06 µg/ml) for 2 h and then
126 infected with SARS-CoV-2 (MOI: 0.01), after that, cells were washed to remove the viral inoculum,
127 and fresh medium was added. Forty-eight hours later, supernatants were harvest. The SARS-CoV-2
128 production was evaluated by adding the supernatants to Vero E6 cells for 1 hour. After incubation,
129 the inoculum was removed, and monolayers were incubated at 37 °C for 72 hours. Then cells were
130 fixed and stained with crystal violet. Virus endpoint titer was determined by Reed-Muench formula
131 and expressed as TCID₅₀/ml. Our results showed that iota-carrageenan markedly inhibits SARS-
132 CoV-2 production in a dose-dependent manner (Figure 1, B and C). No antiviral activity was only
133 observed at the lowest concentration of iota-carrageenan (0.06 µg/ml). Lastly, there was no reduction
134 in virus production with vehicle formulation, suggesting that the iota-carrageenan, not the sample
135 excipient components, inhibited the SARS-CoV-2 replication (Figure 1B). Finally, Figure 1C shows
136 that inhibition of viral production is also observed when pretreatment with carrageenan is applied to
137 the cells, followed by an infection at a higher MOI (0.1).

138 **4 Discussion**

139 Calu-3 cell culture infection has been used as a model to evaluate the activity against SARS-CoV-2
140 of different drugs: suramin (Salgado-Benvindo et al., 2020), nafamostat (Yamamoto et al., 2020),
141 exogenous interferon (Felgenhauer et al., 2020), chloroquine, and hydroxychloroquine (Hoffmann et
142 al., 2020).

143 Recent research has shown that the Vero and Calu-3 cells results do not always coincide. One
144 remarkable example is hydroxychloroquine's antiviral activity when tested in Vero cells, which could
145 not be reproduced on infected Calu-3 cells. These preliminary results, obtained in Vero cells,
146 prompted the premature use of this drug to prevent or treat COVID-19 in patients. Still, the results of
147 clinical trials carried out later have not shown efficacy, in accordance with what was observed in
148 Calu-3 studies.

149 When considering Vero E6 as a model, it should consider their deficient expression ACE2 and
150 TMPRSS2 and the non-specific endocytic viral uptake mechanisms responsible for viral entry in this
151 cell line. Calu-3 cells, besides of the exposed before, are very similar to primary cultures of bronchial
152 epithelium obtained by biopsy or surgery; it develops the characteristic tight junctions present in the
153 respiratory epithelium, expression of cystic fibrosis transmembrane conductance regulator (CFTR)
154 chloride channels, capacity for the secretion of mucus and proteins towards the apical end and
155 exchange of water and electrolytes (Duszyk, 2001; Zhu et al., 2008).

156 In our research, we used dilutions of a commercially available iota-carrageenan spray. The
157 concentrations found to be active in vitro, as shown in previous (Bansal et al., 2020), and in the
158 current work, are those that would be achieved using the spray according to the approved dosage.
159 These results are supporting the efficacy of this spray in the prevention of COVID-19 in a clinical

160 trial recently carried out with frontline healthcare personnel exposed to SARS-CoV-2 (Figuroa et
161 al., 2021).

162 **5 Conclusion**

163 In summary, our results confirm that a formulation of iota-carrageenan and sodium chloride available
164 as a nasal spray effectively inhibited SARS-CoV-2 infection in vitro in human respiratory epithelial
165 cell line culture, strengthening the hypothesis that a nasal spray with iota-carrageenan may be helpful
166 in the prevention or treatment of COVID-19 and reinforces the interest in the development of clinical
167 trials on this topic.

168 **6 Conflict of Interest**

169 The authors declare that the research was conducted in the absence of any commercial or financial
170 relationships that could be construed as a potential conflict of interest.

171 **7 Author Contributions**

172 AC, AVD, and JMF conceptualized the study. AC, AV, CP and AVD developed the methodology.
173 JMF wrote and prepared the original draft. AC, AVD, and CP wrote, reviewed, and edited the
174 manuscript. AC and AVD supervised the study. All authors contributed to the article and approved
175 the submitted version.

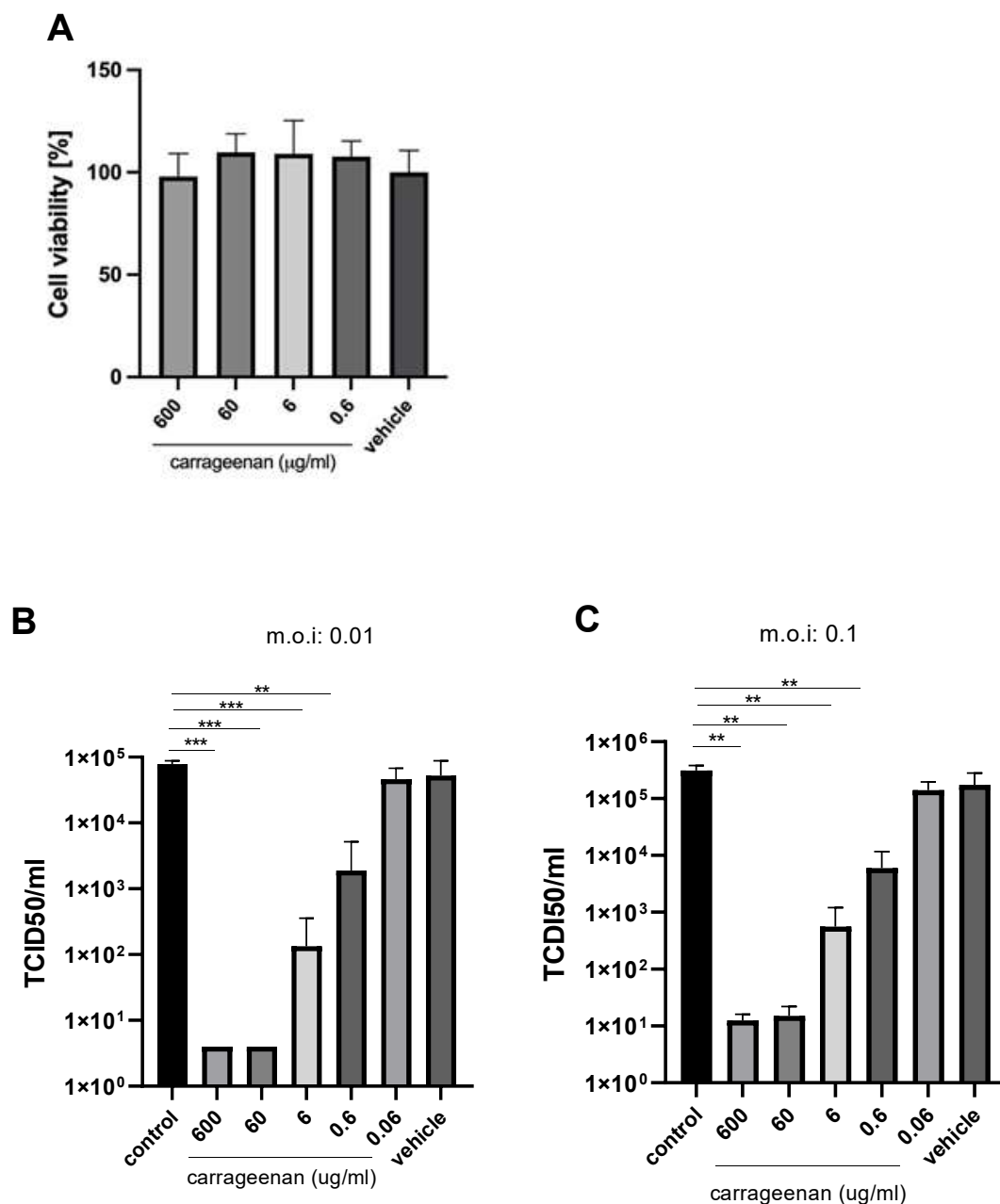
176 **8 Funding**

177 This study was partially supported by the Instituto de Investigaciones Biomédicas en Retrovirus y
178 SIDA (INBIRS), and the Instituto de Ciencia y Tecnología Dr. César Milstein - (Consejo Nacional de
179 Investigaciones Científicas y Técnicas CONICET- Fundación Pablo Cassará).

180

181

182 **Figure 1**



183

184 **Figure 1. A) Cellular viability assays.** Calu-3 cells were treated with iota-carrageenan or vehicle
185 (600 $\mu\text{g/mL}$ to 0 $\mu\text{g/mL}$) for 48 h at 37°C. After incubation, cellular viability was analyzed, and no
186 statistically significant difference was found between the groups compared to the vehicle control
187 group. Data are expressed as mean \pm SD derived from three independent experiments. **B & C)**
188 **Infection assays.** Calu-3 cells were pre-treated with iota-carrageenan or placebo (600 $\mu\text{g/mL}$ to 0
189 $\mu\text{g/mL}$) for 1 h. After one hour of pretreatment, cells were infected, in two different conditions MOI:
190 0.01 (1B) and MOI: 0.1 (1C) with SARS-CoV-2 and incubated for 48 h in the presence of iota-
191 carrageenan. Supernatants were harvested and virus yield. Data are expressed as mean \pm SD derived
192 from three independent experiments.

193 **Table 1. Composition of candidate nasal formulations (samples containing iota-carrageenan)**

Component	Sample	Vehicle
Iota-carrageenan	1.7 mg/mL	-
Sodium Chloride	9 mg/mL	9 mg/mL
pH adjusted to	6.00 – 7.00	6.00 – 7.00

194 **9 References**

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