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NICOTINE UPREGULATES ACE2 EXPRESSION AND INCREASES COMPETENCE FOR SARS-COV-2 IN HUMAN PNEUMOCYTES

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Abstract

The COVID-19 pandemic has a variable degree of severity according to underlying comorbidities and life-style. Several research groups have reported an association between cigarette smoking and increased severity of COVID-19. The exact mechanism of action is largely unclear.

We exposed low ACE2-expressing human pulmonary adenocarcinoma A549 epithelial cells to nicotine and assessed ACE2 expression at different times. We further used the nicotine-exposed cells in a virus neutralization assay.

Nicotine exposure induces rapid and long-lasting increases in gene and protein expression of the SARS-CoV-2 receptor ACE2, which in turn translates into increased competence for SARS-CoV-2 replication and cytopathic effect.

These findings show that nicotine worsens SARS-CoV-2 pulmonary infection and have implication for public health policies.

Introduction

As of September 28, the ongoing COVID-19 pandemic has caused more than 32 million cases and nearly one million deaths worldwide.

In addition to laboratory markers, several underlying clinical risk factors for COVID-19 severity have been identified [1], such as age, diabetes mellitus, systemic arterial hypertension, cardiovascular diseases, chronic obstructive pulmonary disease, chronic liver disease, and cancer [2,3]. All these risk factors are unmodifiable. Among behavioural, modifiable risk factors, the attendance of crowded places without protective equipment is among the most largely accepted. On the contrary, the effect of smoking tobacco (cigarettes, or water pipe), e-cigarettes, or nicotine replacement therapy (NRT) is still debated. Several authors initially reported a protective role for nicotine based on the lower prevalence of current smokers among COVID-19 patients [4-7], supported by *in silico* studies on the interaction between SARS-CoV-2 Spike protein receptor-binding domain and nicotinic receptor (nAChR) sub-type α9 and/orα7 [8,9] or by speculations on the therapeutic use of nicotine agonists [10-12] in the quest for better therapeutics. Also, a negative correlation between the age-standardized prevalence of tobacco smoking and the attack rate [13] and mortality of COVID-19 was reported [14].

Smoking, apart from being a risk factor for tobacco-related diseases [15], can be a direct risk factor for COVID-19 transmission by producing exhaled smoke, coughing or sneezing, aerosols containing SARS-CoV-2 in the surroundings and contaminating surfaces [16], and has been reported to double severity[17] and mortality [18] in patients affected by COVID-19. Extensive evidence shows the negative effects of exposure to nicotine [19, 20]. Indeed, nicotine is identified as an inducer of angiotensin-converting enzyme 2 (ACE2) overexpression, the only recognized receptor of SARS-CoV-2 [21], in the lower airways of current smokers and COPD patients [11, 22-25], suggesting that higher levels of ACE2, i.e.

induced by nicotine exposure, implies more gateways for the SARS-CoV-2 virus. Moreover, it is possible to hypothesize that SARS-CoV-2 entry and replication may be facilitated or induced through mechanisms related to nAChR mediated-pathways, all converging to ACE2 regulation and signalling [15, 25].Additionally, nicotine exposure induces a pro-thrombotic state *via* stimulation of tissue factor (TF) expression both in endothelial cells and smooth muscle cells [26] as well as neo-angiogenesis [27] and, finally, elevated serum cotinine levels, the metabolite of nicotine, are associated with left atrial abnormalities, a possible mechanism for increased risk of cardiovascular diseases (CVD) [28]. Importantly, post-mortem examinations of people who died with COVID-19 revealed widespread vascular abnormalities including thrombosis, microangiopathy, and a high degree of angiogenesis [29]. Taking into consideration all the above findings and the observation that nicotine potentiates viral infections [30], it is unlikely that smoking or vaping may offer therapeutic benefits in COVID-19.

Nevertheless, the underlying mechanism of action through which nicotine could facilitate SARS-CoV-2 infection remains largely unknown. Evidence on the regulation of ACE2 in the lungs after exposure to nicotine is still inconclusive, with evidence both for down-regulated ACE2 axis [31], in bronchial epithelial cells [32] and for enhanced ACE2 expression in lung tissue of COPD patients, at a lower extent in healthy smokers, and absent in healthy non-smoking individuals [23, 33-35].

It is important to remark that until now there is no indication that nicotine prevents the entry of SARS-CoV-2 into host cells or that protects cells from the severity of SARS-CoV-2 infection, for this reason, we planned studies where cells were infected with SARS-CoV-2 in the presence or absence of nicotine.

We investigated here whether ACE2 is overexpressed in pneumocytes after exposure to nicotine, and if this leads in turn to increased SARS-CoV-2 replication and cytopathic effect. effect.

Materials and methods

Cells exposure to Nicotine

The Nicotine (Sigma-Aldrich, St. Louis, MI, USA) was assayed in *vitro* experiments for its ability to stimulate ACE2 expression in A549 cells. The A549 type II pulmonary adenocarcinoma cell line (isolated in 1973, [36]) was selected because of the low-levels of ACE2 expression. A549 cells require transgenic ACE2 expression to be used in plaque reduction neutralization test (PRNT), thus it is the ideal cell line to measure ACE2 up-regulations.

In vitro cytotoxicity of Nicotine and cell viability assay

For determining the optimal experimental dose of nicotine and for evaluating the proliferation of the cells under nicotine treatment, the viability of the pulmonary A549 cells was assessed. Cytotoxicity was determined by the alamarBlue® assay (Thermo Fisher Scientific, Waltham, MA, USA).

Anchorage-independent soft-agar colony formation and cell migration

Anchorage-independent growth was assayed by the ability of cells to form colonies in soft agar. Colonies were visualized and quantified under a microscope after 18 days of cultivation, with or without nicotine at concentrations of 0.1 or 0.01 μM, re-placed every 48 h. At the end of the incubation period, colonies were stained with 0.01% crystal violet in 70% ethanol, then photographed and counted under an inverted microscope.

CytoSelect® 24-Well Cell Migration Assay (8 μm, Fluorometric format) was obtained by Cell Biolabs [catalog number CBA-101-C (San Diego, USA)], and experiments were performed according to the manufacture's protocol.

Real-time PCR for ACE2 mRNA

Total RNA was extracted from A549 cells by using TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) and then used for quantitating ACE2 and β-Actin gene RNAs using real-time PCR assays. The assays were carried out on a Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using commercially one-step Quantitect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, California, USA) with the following primer pairs: (i) ACE2 forward: 5′- GGGATCAGAGATCGGAAGAAGAAA -3′ and ACE2 backward: 5′-AGGAGGTCTGAACATCATCAGTG-3′; (ii) β-Actin forward: 5′-AAGGAGAAGCTGTGCTACGTC-3′ and β-Actin backward: 5′-AGACAGCACTGTGTTGGCGTA-3′. The assays were performed using a previously standardized thermal program (50.0 °C for 30 min, 95 °C for 15 min, 35 cycles of 15 s at 94.0 °C, 60 °C for 30 s, and 72 °C for 30 s). Melting curves were produced by plotting the fluorescence intensity against the temperature as the temperature was increased from 65.0 to 95.0 °C at 0.5 °C/s. All samples were amplified in triplicate and negative PCR controls with no RNA template was included in each round of tests. Bio-Rad CFX Maestro 1.1 software was used to analyse the data (Bio-Rad Laboratories, Hercules, CA, USA). Differences in ACE2 expression were represented as the fold change in gene expression using the $2^{-\Delta\Delta CT}$ method. The housekeeping gene β-Actin was used as the internal control for normalization following preliminary experiments with Nicotine and throughout the study was found not to be appreciably modulated.

ACE2 protein detection

ACE2 was measured with Human ACE2 ELISA Kit ab235649 (www.abcam.com/human-ace2-elisa-kit-ab235649.html) according to manufacture instructions. Cells were treated with Nicotine at 0.01 µM for 1 or at 0.1 µM for 1, 24 h, or 48 h.

Western blotting

Cells were treated with Nicotine at 0.1 µM for 1 or 24 h. The ACE2 protein was detected by a mouse anti-ACE2 monoclonal antibody raised against amino acids 631-805 of ACE2 of human origin [ACE2(E-11): sc-390851, Santa Cruz Biotechnology Incorporation, Dallas, USA] or with anti-α7-nAChR NBP1-52375 (Novus Biological, Cambridge UK), β-actin (1:2500, ab20272, Abcam). Membranes were incubated with Horseradish peroxidase-labelled-conjugated secondary antibody corresponding to the primary antibody in the blocking buffer for 1 h at room temperature. An enhanced chemiluminescence kit (Western blot detection reagent, GE Healthcare UK Limited) was used for the detection of recognized proteins. Densitometric analysis for quantification of the relative level of protein expression was performed using Amersham Image Quant800 (EG Healthcare) with software ImageQuant TL 7.0.

SARS-CoV-2 culture

As inoculum was used a SARS-CoV-2 positive cell supernatant. The cell supernatant, obtained by infecting Vero E6 cells with a nasopharyngeal swab of a patient with SARS-CoV-2 infection, contained 2,000 tissue culture infectious dose 50 ($TCID_{50}$), as estimated by endpoint titration.

Adherent A549 cell lines, grown in appropriate conditions of temperature and medium supplemented with heat-inactivated FBS and antibiotics/antimycotics, were exposed to the viral inoculum (0.5 ml) in the form of traditional culture when cell monolayers were less than 2 days old. After infection, the inoculum was removed, the monolayers rinsed three times with sterile phosphate-buffered saline (PBS), and 3 ml of the appropriate culture medium was added. All culture plates were incubated in a humidified 37°C incubator in an atmosphere of 5% CO2. Cells were monitored daily for the development of cytopathic effects (CPE). After three days, 400 µl of cell supernatant was used for total nucleic acid extraction for SARS-

CoV-2 RNA testing. Negative control samples were supernatants obtained from A549 cells not infected with SARS-CoV-2.

SARS-CoV-2 immunofluorescence assay

The presence of SARS-CoV-2 antigens in A549 infected cells was evaluated by indirect immunofluorescence (IF) using polyclonal antibodies directed against N and S proteins of SARS-CoV-2 (Sino Biological, Beijing, China). For the IF assay, the cells grown in an 8-well chamber slide were rinsed twice with PBS, fixed in methanol solution at room temperature for 30 min, washed three times with PBS, and then incubated with the specific anti-SARS-CoV-2 antibodies (diluted 1:60 in PBS) for 1 hour at 37°C. After 3 washes with PBS, the secondary antibody consisted of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:1000 in PBS; Sigma, St. Louis, MI, USA) was added for 1 hour at 37 °C. After three final washings, drying, and mounting, the slides were observed under a fluorescence microscope.

SARS-CoV-2 RNA detection

SARS-CoV-2 genome was detected by extracting RNA from 400 ml of cell supernatant using the Nimbus platform (Hamilton, Reno, NV, USA). After extraction, viral RNA was amplified by using the Allplex™ 2019-nCoV assay (Seegene, Seoul, South Korea) on the CFX96 instrument (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The real-time Allplex™ 2019-nCoV assay simultaneously detects the N gene, the E gene, and the RdRP gene of the SARS-CoV-2 genome. The cycle threshold (Ct) value was recorded for each of the 3 genes. Samples were considered positive when a PCR signal was detected at C_t<40 for any gene.

Results

Nicotine stimulates the expression of the ACE2 gene

In all experiments, A549 cell lines were treated for 24 hours with 0.1 μM of nicotine and then evaluated for ACE2 mRNA expression by reverse transcription real-time PCR. This timing was selected in previous experiments because did not induce cytotoxicity on A549 cells (Figure 1) but induced the highest level of ACE2 mRNA stimulation for all the nicotine concentrations investigated over the 48 hours of incubation that were explored (Figure 2). The negative control was cell cultures exposed to the diluent alone in which the nicotine was solubilized (e.g. 1 μl ethanol in 1 ml complete DMEM). As shown in Figure 2, nicotine added at 3 different doses produced a marked increase (nicotine dose: 0.1 μM), moderate increase (nicotine dose: 0.01 μM), or no detectable effect (nicotine dose: 10 μM) on expression levels of the ACE2 gene relative to the levels found in the negative control cultures. The increase in the gene expression peaked after 24 hours of exposure and then declined at 48 hours.

Nicotine stimulates the secretion of ACE2 protein and induces cell proliferation and migration

In these experiments, we measured the quantitative levels of ACE2 protein in extracts of A549 cell lines exposed to nicotine by using Western Blot analysis and a sandwich ELISA. As shown in Figures 3, results obtained by both methods revealed that the treatment with nicotine-induced levels of ACE2 protein was greatly enhanced in comparison to negative controls. HeLa cells are negative controls since according to Zhou et al. [37] do not express ACE2 and consequently are not infected by SARS-CoV-2. The increase in the protein expression peaked after 24 hours of exposure and then declined at 48 hours as observed for gene expression. As shown in Figure 4, panels A and B, treatment of cells with nicotine increased both the number and the size of the colonies as well as the migration of A549 cells in a dose-dependent manner; the maximum effect was observed at 0.1μM

Nicotine facilitates the in vitro replication of SARS-CoV-2

Given the results shown above, we examined whether the treatment with Nicotine could increase SARS-CoV-2 replication in A549 cell cultures. In these experiments, A549 cell cultures previously exposed for 24 hours to Nicotine at the dose of 0.1 μM were then infected with 2 $TCID_{50}$ of SARS-CoV-2. This $TCID_{50}$ dose was selected according to results from preliminary experiments in which 100, 20, and 2 $TCID_{50}$ doses were tested. Two $TCID_{50}$ of SARS-CoV-2 was the optimal dose for investigating differences in virus replication on A549 cell cultures. No or slight differences were observed when 100, and 20 $TCID_{50}$ doses were used, respectively. The increase in ACE2 expression induced an increased SARS-CoV-2 replication as detected by quantitative real-time PCR targeting three different viral genes (Figure 5), and by an immunofluorescent assay for viral proteins (Figure 6), which revealed an increased cytopathic effect (Figure 7).

Discussion

In this work, we report data supporting our initial hypothesis that nicotine may be the "hidden" link of SARS-CoV-2 infection [24]. Specifically, our data show that nicotine:*(i)* enhances the expression levels of α7-nAChR in A549 human adenocarcinoma cell line; *(ii)*is not cytotoxic and induces cell proliferation, as well as cell migration; *(iii)* up-regulates ACE2 expression; *(iv)* increases SARS-CoV-2 replication;*(v)* increases the transcription of SARS-CoV-2 viral proteins; and *(vi)* increases SARS-CoV-2 cytopathic effect. These results address our research question and robustly support the hypothesis that there is a causal link between exposure to nicotine and the involvement and severity of Covid-19 infection. The concentration of nicotine in all the experiments is principally 0.1 μM, cells are treated both after a short time (1 h) and for a longer time (24-48 h) mimicking the human exposure in smokers. The percentage of nicotine in one tobacco cigarette is approximately 1-2% =1-2 g/100. Considering a human body weight average equal to 68 kg one cigarette may deliver approximately 10-30 μg/Kg, resulting in a peak

plasma level of 10-50 ng/ml. A concentration equal to 50 ng/ml can be converted to molarity dividing by nicotine MW (i.e. 162) [50 ng/ml divided by 162] =0.309 =$3.1x10^{-7}$ M [38].

To the best of our knowledge, this is the first work that directly shows the effect of nicotine on SARS-CoV-2 infection. Nicotine may affect different processes in A549 cells, such as growth, invasion, and metastasis, epithelial-mesenchymal transition (EMT), induction of angiogenesis factor, inhibition of drug-induced apoptosis, reduction of p53, and phosphop53 [25, 27]. Importantly, nicotine in A549 cells, as well as in human unaffected bronchial epithelial cells [22, 24] enhances the expression of ACE2 both as mRNA and as proteins. Physiologically, ACE2, in the renin-angiotensin system (RAS), promotes the degradation of Angiotensin (Ang) II, potent vasoconstriction, pro-inflammatory, and pro-fibrosis agent, converting Ang II into Ang(1-7), a vasodilating, anti-proliferative, and apoptotic agent [39]. ACE2 is the only well-recognized functional receptor of SARS-CoV-2, that allows viral entry into human cells [21, 37, 39]. A specific region of SARS-CoV-2 namely spike protein (S1) interacts with ACE2 and in cooperation with host proteases, mainly transmembrane serine protease 2 (TMPRSS2), promotes cellular entry into host cells. Various experiments support the role of ACE2 in the entry of SARS-CoV-2, thus antibodies against ACE2 block the SARS-CoV infection [39], while expression of ACE2 in refractory cell lines (i.e. HeLa cells) results in SARS-CoV-2 replication [37]. After binding of SARS-CoV-2 to ACE2, the membrane fusion and virus entry inside the cell, ACE2 is down-regulated thus, the degradation of Ang II is attenuated or blocked. The comprehensive understanding of the mechanisms involved in the nicotine-up regulation of ACE2 pulmonary expression is still unknown. Among these, there is a possible role of nAChR, engaged by nicotine. We have recently shown that nicotine, through activation of α7-nAChR, increases the expression level of ACE2 in unaffected human bronchial epithelial cells (HBEpC) inducing phospho-S6 ribosomal protein (Ser235/236), Akt1, phospho-Akt (Ser473 and

Thr308) and phospho-p44/42 MAPK (Thr202/Tyr204) [24, 25]. No increase of ACE2 is observed in si-mRNA-α7-HBEpC or the presence of α-bungarotoxin, an α7 nicotine antagonist, suggesting that the increase of ACE2 is specifically mediated by α7-nAChR [24, 25]. Very recently, Leung et al. 2020 [22], in a cohort of 42 subjects reveal evidence in support of this hypothesis finding that CHRNA7, encoding α7-nAChR, expression in airway epithelial cells is significantly correlated with the expression of ACE2. CHRNA7 expression is higher in COPD than in non-COPD patients with a trend in the direction of higher expression in current smokers compared to former and never smokers. Cai *et al* [40] reported higher ACE2 gene expression in former smoker's lungs compared to non-smoker's lungs. Also, they found higher ACE2 gene expression in Asian current smokers compared to non-smokers but not in Caucasian current smokers, which may indicate the presence of gene-smoking interaction. Also, they found that the ACE2 gene is expressed in specific cell types related to smoking history and location. In bronchial epithelium, ACE2 is actively expressed in the goblet cells of current smokers and club cells of non-smokers. In alveoli, ACE2 is actively expressed in remodelled AT2 cells of former smokers [40]. Accordingly, sub-chronic e-cigarette exposure with Nicotine increased inflammatory cellular influx of macrophages and T-lymphocytes in mice, including increased pro-inflammatory cytokines in bronchoalveolar lavage and increased SARS-CoV-2 ACE2 receptor, whereas nAChRα7 KO mice show reduced inflammatory responses associated with decreased ACE2 receptor [41].

Lee *et al* reported, previously, that cigarette smoking, but not vaping, up-regulates ACE2 [42]. We show here instead of that nicotine (and hence potentially any nicotine-delivering habit) can up-regulate ACE2 expression in pneumocytes.

Nevertheless, some publications sustain that nicotine may be a protective factor against COVID-19 infection [4]. Specifically, Changeux *et al*.[43] hypothesized that the nAChR

may be the target for the prevention and control of Covid-19 infection. The authors conclude their paper by noting "…*under controlled settings, nicotinic agents could provide an efficient treatment for an acute infection such as Covid-19*." This assertion is the base of the so-called "Nicotinic Hypothesis" [43]. Changeux *et al.*[43] do not accept the generally well-confirmed finding that SARS-CoV-2 enters human cells through ACE2, and in turn, does not agree that nicotine up-regulates ACE2 expression. Thus Changeux *et al.*[43] claim that α7-nAChR may play a key role in SARS-CoV-2 infection, being the receptor of SARS-CoV-2, and consequently argue that nicotine may compete with or even block the binding of SARS-CoV-2 to nAChR. Indeed, according to this hypothetical mechanism, nicotine may reduce smokers' chance of infection. This hypothesis, although captivating, until now is not supported by any experimental data. However, it has been shown with *in silico* experiments that the SARS-CoV-2 S protein binds to α7-nAChR [10] or with both α7- and α9-nAChR [9]. These findings are not verified *in vivo* and *in vitro*. Specifically, SARS-CoV-2 has not been reported yet to open the nAChR channel and to allow the entry of Ca^{2+}inside a cell, as well-known for Acetylcholine and nicotine [27]. Moreover, no SARS-CoV-2 binding to nAChR is studied in Acetylcholine binding protein (AChBP) crystal structures. Our data, summarized in Figure 8, show that in the presence of nicotine, when α7-nAChR are up-regulated and engaged (i.e. ERK and MAPK activation), ACE2 is more expressed and in turn, the infection property of SARS-CoV-2 is increased. A549 cell line is broadly utilized as a model of type II pulmonary epithelial cell, however, these cells are incompatible with SARS-CoV-2 infection and need ACE2 transfection to become permissive to virus infection [44]. In our experiments, we show that nicotine converts A549 cells to a permissive state for SARS-CoV-2 infection. We hypothesize that this conversion is linked to an increased expression of ACE2. However, it is possible that other co-receptors possibly stimulated by nicotine may be involved. It has been known that nicotine not only increases cell proliferation [this work and reviewed in 27]

but prevents or delays cell death [25] generating more functional cells available for a possible viral infection. Thus, SARS-CoV-2 speedily replicates in actively transcriptional cells [45] requiring the availability of cellular nucleotide pools [46], and in turn of proliferating and viable cells.

It has been reported that the so-called "cytokine storm" is associated with COVID-19 severity and is an important cause of COVID-19 death [47]. Indeed, intracellular SARS-CoV-2 triggers a strong immune response accompanied by inflammatory cytokine production (i.e. TNF-α and IL-6) [47]. Literature data report that nicotine reduces the release of TNF-α and IL-6 after cell stimulation with LPS in human bronchial epithelial cells [48]. Experiments were conducted on twelve adult male normal subjects of which six received overnight transcutaneous nicotine administration by application of a standard patch (7 mg) and then six hours later, all subjects were given an intravenous dose of endotoxin (2 ng/kg). After an additional 24 h, all subjects were evaluated for cytokines expression. No significant differences in the circulating TNF-α, IL6, and IL8 levels between the nicotine and placebo groups [49]. We induced cytokines production, for the first time in human unaffected cells, i.e. HBEpC, using Polyinosinic-polycytidylic acid (poly(I:C)), a synthetic analogue of double-stranded RNA (dsRNA), which mimics viral infection and induces antiviral immune responses by promoting the production of both Type I Interferon and inflammatory cytokines such as TNF-α and IL6. Nicotine reduces the amount of TNF-α (- 37.3%) and IL-6 (- 29.3%) in these cells [25]. These data suggest that the reduction of cytokines by nicotine is not very strong and question its use to control the cytokine storm.

Several epidemiological surveys have been published or are available as pre-print. Evidence from these studies is somehow contrasting, and the role of tobacco smoking, NRT, or vaping is still to be assessed [4-7, 50-57]. In many cases, the presence of

underreporting in recording smoking history at admission, especially to ICU during the COVID-19 outbreak, when the hospitals are overwhelmed, cannot be ruled out. To clarify the role of tobacco smoking on COVID-19 severity and progression, a longitudinal observational study titled COvid19 and SMOking in Italy (COSMO-IT) was designed [58]. One of the objectives of the study is to quantify the role of tobacco smoking and smoking cessation on the severity and progression of COVID-19 in hospitalized patients.

Nicotine exposure, through different types of smoking habits, sustains cellular and molecular mechanisms for SARS-CoV-2 susceptibility and severity throughout the infection in the lungs as well as in other organ systems expressing both α7-nAChR and ACE2 (i.e. neurons, enterocytes). Together, our data help to better understand and characterize the connections between airway epithelial ACE-2, and α7-nAChR, and the development of severe COVID-19. In the light of present results caution shall be applied in trials utilizing nicotine to prevent or treat COVID-19.

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Legends to Figures:

Figure 1. Cytotoxicity of nicotine on A549 cell cultures

Figure 2. ACE2 gene expression in A549 cells at different times after exposure to different doses of nicotine, measured by real-time PCR. The mock group is represented by cell cultures exposed to the diluent alone in which the Nicotine was solubilized. Differences in ACE2 expression are represented as the fold change in gene expression using the housekeeping gene β-Actin as the internal control for normalization.

Figure 3. Quantitative levels of α7-nAChR and ACE2 proteins in extracts of A549 and HeLa cells exposed to nicotine for 1 or 24 h at 0. 1 μM by using Western Blot analysis (Panel A and B densitometric analysis). Results of two different experiments are reported. All data are expressed as mean ± SD of three independent experiments. (Panel C) Quantitative levels of ACE2 proteins in A549 cells exposed to 0.01 for 24 h or 0.1 μM for 1, 24, or 48 h evaluated by sandwich ELISA. Regression equation linearity for ACE2 human standard was performed with Prism. All data are expressed as mean ± SD of two independent experiments performed in triplicate. Statistical significance was analyzed with one-way ANOVA with multiple-comparison and post hoc test with Bonferroni correction. Experiments are performed at least two times in triplicate.

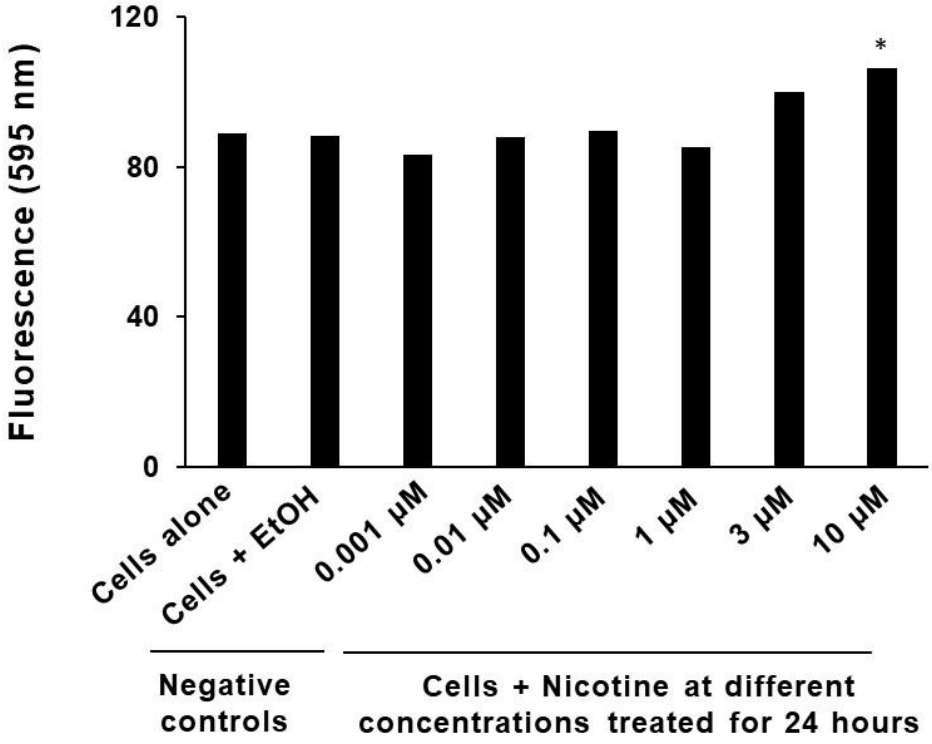
Figure 4. Panel A. Anchorage-independent growth. Effects of treatment with nicotine 0.01 or 0.1 μM on A549 cells. Panel A: Colony formation. Magnification 20x or 40x. Panel B Cell migration. 30,000 A549 cells were plated and treated with different concentrations of nicotine 0.01 or 0.1 μM for 24 h. All data are expressed as mean ± SD of three experiments performed in triplicate. . Statistical significance was analyzed with one-way ANOVA with multiple-comparison and post hoc test with Bonferroni correction. Experiments are performed at least two times in triplicate.

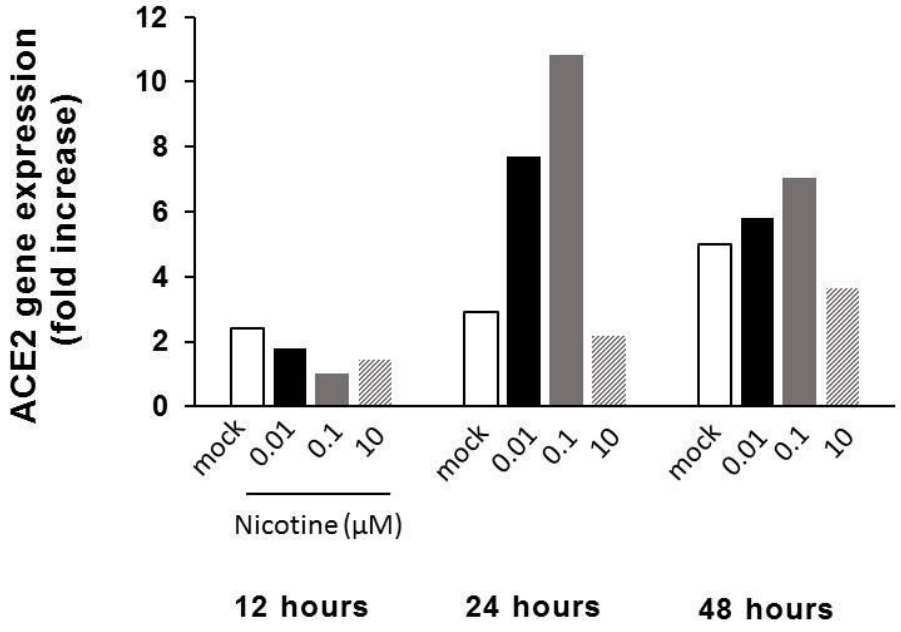
Figure 5. Cycle threshold analysis of 3 different PCR targets (E, RdRp, and N genes) of the SARS-CoV-2 genome in the supernatants of A549 cells previously exposed to nicotine.

Figure 6. Immunofluorescence Assay for SARS-CoV-2 S and N proteins before or after challenge with nicotine.

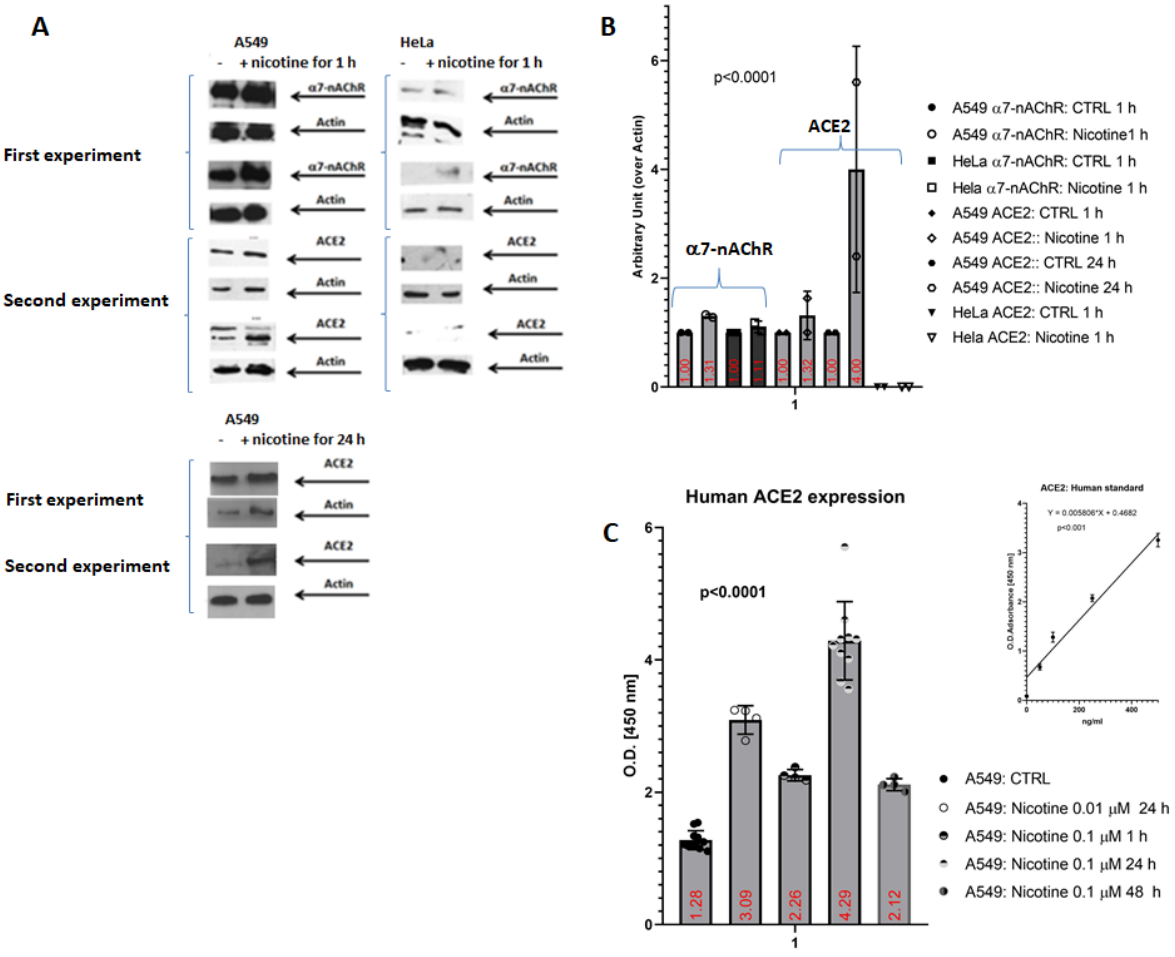
Figure 7. Increase in the cytopathic effect of SARS-CoV-2 on A549 cells previously exposed to nicotine. More than 70% increase in cell mortality is observed.

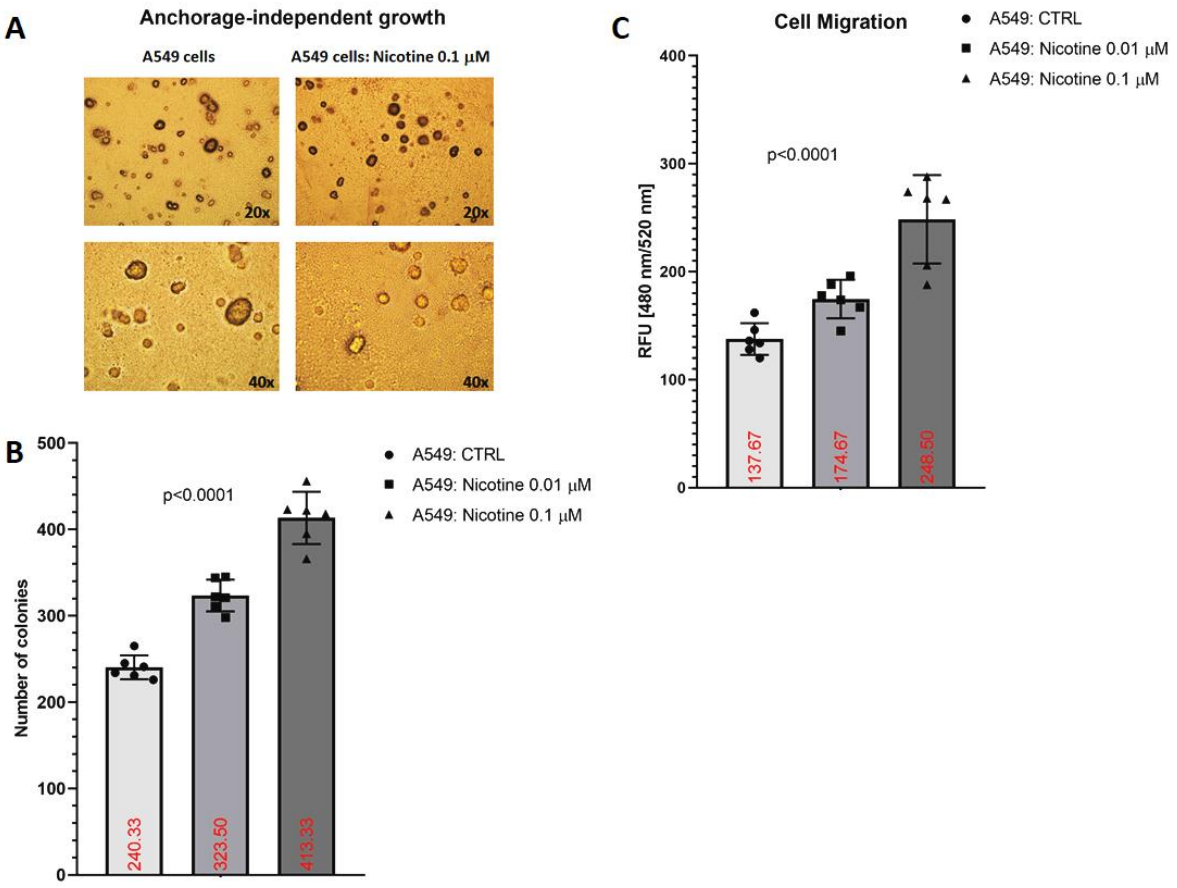
Figure 8.A schematic model for how nicotine exposure may work on the risk of COVID-19. On the left in non-smokers (not exposed to nicotine). On the right in smokers exposed to nicotine.

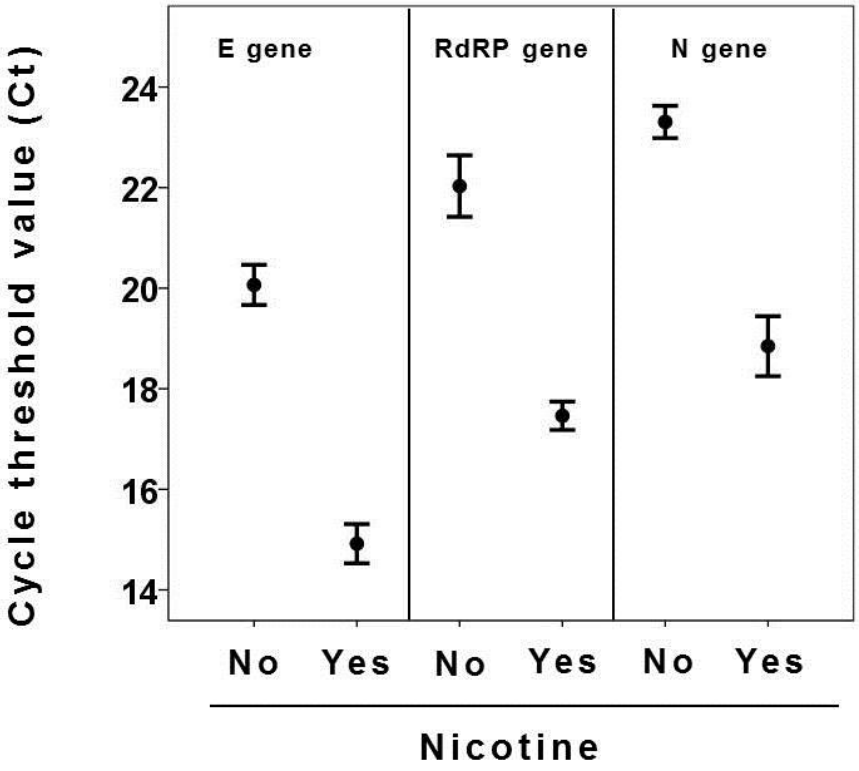

Fluorescence (595 nm)
120
80
40
0
*
Cells alone
Cells + EtOH
0.001 µM
0.01 µM
0.1 µM
1 µM
3 µM
10 µM
Negative controls
Cells + Nicotine at different concentrations treated for 24 hours

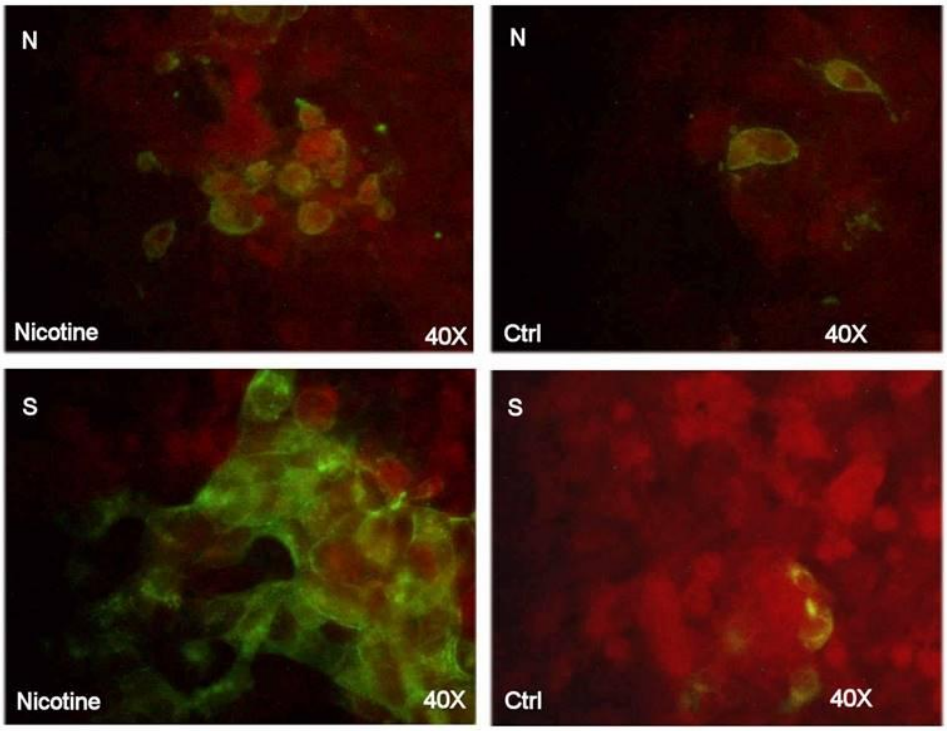

ACE2 gene expression
(fold increase)
12
10
8
6
4
2
0
mock
0.01
0.1
10
mock
0.01
0.1
10
mock
0.01
0.1
10
Nicotine (μM)
12 hours
24 hours
48 hours

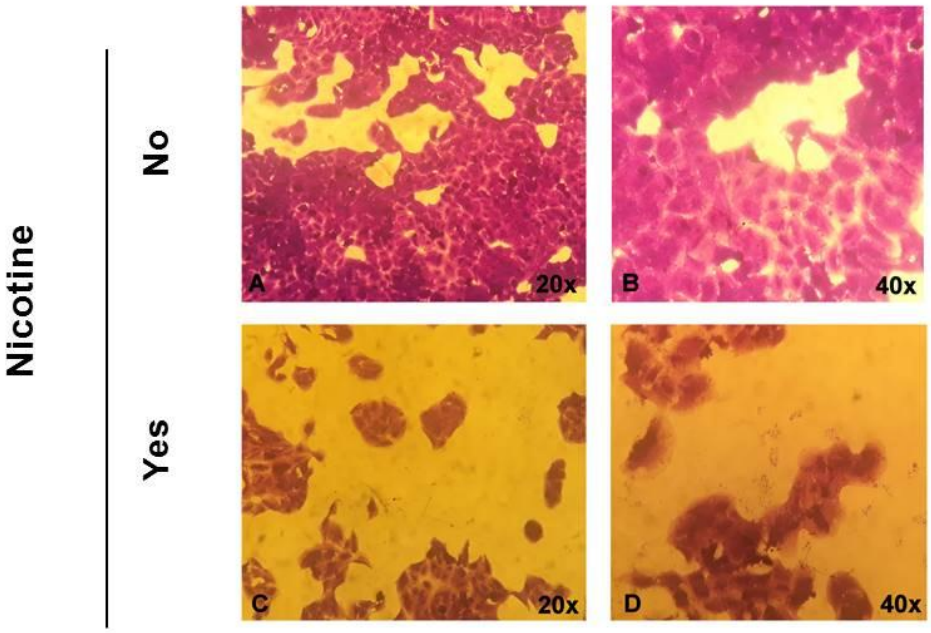
α7-nAChR and ACE2 proteins expression

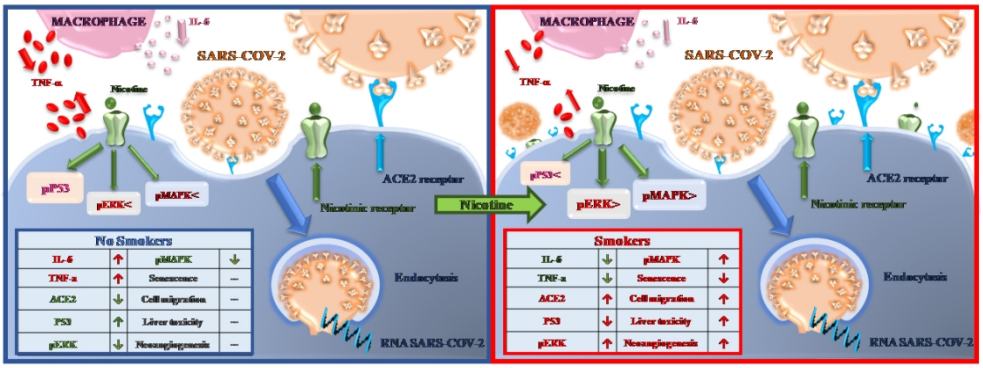



A
Anchorage-independent growth
A549 cells
A549 cells: Nicotine 0.1 μM
20x
20x
40x
40x
B
p<0.0001
A549: CTRL
A549: Nicotine 0.01 μM
A549: Nicotine 0.1 μM
Number of colonies
500
400
300
200
100
0
240.33
323.50
413.33
C
Cell Migration
A549: CTRL
A549: Nicotine 0.01 μM
A549: Nicotine 0.1 μM
p<0.0001
RFU [480 nm/520 nm]
400
300
200
100
0
137.67
174.67
248.50


E gene
RdRP gene
N gene
Cycle threshold value (Ct)
24
22
20
18
16
14
No
Yes
No
Yes
No
Yes
Nicotine


N
Nicotine
40X
N
Ctrl
40X
S
Nicotine
40X
S
Ctrl
40X


Nicotine
No
Yes
A
20x
B
40x
C
20x
D
40x


MACROPHAGE
IL-6
SARS-COV-2
TNF-α
Nicotine
pP53
pERK<
pMAPK<
ACE2 receptor
Nicotinic receptor
Endocytosis
RNA SARS-COV-2
No Smokers
IL-6 ↑ pMAPK ↓
TNF-α ↑ Senescence --
ACE2 ↓ Cell migration --
P53 ↑ Liver toxicity --
pERK ↓ Neoangiogenesis --
Nicotine
MACROPHAGE
IL-6
SARS-COV-2
TNF-α
Nicotine
pP53<
pERK>
pMAPK>
ACE2 receptor
Nicotinic receptor
Endocytosis
RNA SARS-COV-2
Smokers
IL-6 ↓ pMAPK ↑
TNF-α ↓ Senescence ↓
ACE2 ↑ Cell migration ↑
P53 ↓ Liver toxicity ↑
pERK ↑ Neoangiogenesis ↑

Materials and methods

Cells exposure to Nicotine

The Nicotine (Sigma-Aldrich, St. Louis, MI, USA) was assayed in *in vitro* experiments for its ability to stimulate ACE2 expression in A549 cells. The compound provided as a liquid was solubilized in absolute ethanol at the final stock concentration of 0.1 mM according to the manufacturer's recommendations and then stored in aliquots at room temperature away from light source.For cell stimulation, fresh medium was prepared by adding Nicotine at the final concentrations of 0.1 µM to Gibco ™ Dulbecco's Modified Eagle (DMEM; Paisley, Scotland,UK) medium supplemented with 1% glutamine and 10% foetal bovine serum (FBS). The A549 type II pulmonary adenocarcinoma cell line (isolated in 1973, [26]) was selected because of the low-levels of ACE2 expression. The stimulation tests were carried out by exposing A549 cells in 3 ml of complete DMEM in 6-well plates at 37°C in 5% CO_2 to the indicated concentrations of Nicotine. At the end of the incubation period, cells and culture supernatants were transferred to centrifugation tubes and separated at 500g for 10 min. The cells were then stored as such at -80 °C until assayed for ACE2 expression. The Nicotine was assayed in triplicate wells in at least two independent experiments, and the wells were tested individually.

In vitro cytotoxicity of Nicotine and cell viability assay

For determining the optimal experimental dose of Nicotine and for evaluating the proliferation of the cells under Nicotine treatment, viability of the pulmonary A549 cells was assessed. Cytotoxicity was determined by the alamarBlue® assay (Thermo Fisher Scientific, Waltham, MA, USA). The assay incorporates a fluorometric/colorimetric growth indicator based on detection of metabolic activity. Specifically, the system incorporates an oxidation-reduction (REDOX) indicator that both fluoresces and changes colour in response to chemical reduction of growth medium resulting from cell growth. The active ingredient is resazurin (IUPAC name: 7-hydroxy-10-oxidophenoxazin-10-ium-3-one), which is water-soluble, stable in culture medium, is non-toxic and permeable through

cell membranes. As cells being tested grow, innate metabolic activity results in a chemical reduction of alamarBlue®. Continued growth maintains a reduced environment while inhibition of growth maintains an oxidized environment. Reduction related to growth causes the REDOX indicator to change from oxidized (non-fluorescent, blue) form to reduced (fluorescent, red) form. Data were collected monitoring fluorescence at 595 nm. Briefly, the A549 cells were seeded into 96-well microtiter plates in three replicates and left for 24 hours to adhere. Then, the culture medium was removed and replaced with a fresh one, which contained different concentrations of Nicotine followed by incubation for 12, 24 or 48 hours. Fresh media alone or containing 1µl ethanol was also added to some wells to serve as negative controls. After incubation, Nicotine was removed and the cell monolayers were incubated at 37°C, 5% CO2 for four hours.At the end of the incubation, alamarBlue® was added to each well. Plates were then incubated for 1 hour at 37°C and checked for colour change.

Anchorage-independent soft-agar colony formation

Anchorage-independent growth was assayed by the ability of cells to form colonies in soft agar. The bottom agar consisted of a growth medium containing 10% FBS and 0.75% agarose in 60-mm tissue culture dishes. A total of 500 cells were re-suspended in growth medium containing 10% FBS and 0.75% agarose and plated on top of the bottom agar. The cells were incubated at 37°C in 5% CO_2. Colonies were visualized and quantified under a microscope after 18 days cultivation, with or without Nicotine at concentrations of 0.1 or 0.01 µM, re-placed every 48 h. At the end of the incubation period, colonies were stained with 0.01% crystal violet in 70% ethanol. After carefully removing the crystal violet and rinsing with water, colonies were photographed and counted under an inverted microscope.

Real-time PCR for ACE2 mRNA

Total RNA was extracted from A549 cells by using TRIzol reagent (Invitrogen GmbH, Karlsruhe, Germany) and then used for quantitating ACE2 and β-Actin gene RNAs using real-time PCR

assays. The assays were carried out on a Bio-Rad CFX96 real-time PCR system (Bio-Rad Laboratories, Hercules, CA, USA) using commercially one-step Quantitect SYBR Green RT-PCR kit (Qiagen Inc., Valencia, California, USA) with the following primer pairs: (i) ACE2 forward: 5′-GGGATCAGAGATCGGAAGAAGAAA -3′ and ACE2 backward: 5′-AGGAGGTCTGAACATCATCAGTG-3′; (ii) β-Actin forward: 5′-AAGGAGAAGCTGTGCTACGTC-3′ and β-Actin backward: 5′-AGACAGCACTGTGTTGGCGTA-3′. The assays were performed using a previously standardized thermal program (50.0 ℃ for 30 min, 95 ℃ for 15 min, 35 cycles of 15 s at 94.0 ℃, 60 ℃ for 30 s and 72 ℃ for 30 s). Melting curves were produced by plotting the fluorescence intensity against the temperature as the temperature was increased from 65.0 to 95.0 ℃ at 0.5 ℃/s. All samples were amplified in triplicate and negative PCR controls with no RNA template was included in each round of tests. Bio-Rad CFX Maestro 1.1 software was used to analyse the data (Bio-Rad Laboratories, Hercules, CA, USA). Differences in ACE2 expression were represented as the fold change in gene expression using the $2-\Delta\Delta CT$ method. The housekeeping gene β-Actin was used as the internal control for normalization following preliminary experiments with Nicotine and throughout the study was found not to be appreciably modulated.

ACE2 protein detection

ACE2 was measured with Human ACE2 ELISA Kit ab235649 (www.abcam.com/human-ace2-elisa-kit-ab235649.html) according to manufacture instructions.

Western blotting

Cells were treated with Nicotine at 0.1 μM for 1 h then cells were scraped from culture dishes, and protein lysates were mixed with an equal volume of 2× Laemmli's buffer, boiled for 10 min at 100℃, and then resolved by SDS-PAGE on an 8% gel using a Mini-Gel apparatus (Bio-Rad). Subsequently, the proteins were electrophoretically transferred to a nitrocellulose membrane (Bio-Rad). The membranes were blocked with a 5% non-fat dry milk solution in TBS with 0.1% Tween

20 (TBS-T, pH 7.6) for 1 h at room temperature and then incubated in primary antibody dissolved in the blocking solution at 4°C overnight. The ACE protein was detected by a mouse anti-ACE monoclonal antibody raised against aminoacids 631-805 of ACE2 of human origin [ACE2(E-11): sc-390851,Santa Cruz Biotechnology Incorporation, Dallas, USA] or with), anti-α7-nAChR NBP1-52375 (Novus Biological, Cambridge UK), β-actin (1:2500, ab20272, Abcam). After the membranes were washed three times for 5 min in TBS-T, they were incubated with Horseradish peroxidase-labelled-conjugated secondary antibody corresponding to the primary antibody in the blocking buffer for 1 h at room temperature. After three washes, an enhanced chemiluminescence kit (Western blot detection reagent, GE Healthcare UK Limited) was used for the detection of recognized proteins. Densitometric analysis for quantification of the relative level of protein expression was performed using Amersham Image Quant800 (EG Healthcare) with software ImageQuant TL.

SARS-CoV-2 culture

As inoculum was used a SARS-CoV-2 positive cell supernatant. The cell supernatant, obtained by infecting VERO E6 cells with a nasopharyngeal swab of a patient with SARS-CoV-2 infection, contained 2,000 tissue culture infectious dose 50 ($TCID_{50}$), as estimated by endpoint titration.

Adherent A549 cell lines, grown in appropriate conditions of temperature and medium supplemented with heat-inactivated foetal bovine serum and antibiotics/antimycotics, were exposed to the viral inoculum (0.5 ml) in the form of traditional culture when cell monolayers were less than 2 days old. After infection, the inoculum was removed, the monolayers rinsed threetimes with sterile phosphate-buffered saline (PBS), and 3 ml of the appropriate culture medium was added. All culture plateswere incubated in a humidified 37°C incubator in an atmosphere of 5% CO2. Cells were monitored daily for the development of cytopathic effects (CPE). After three days, 400 µl of cell supernatant was used for total nucleic acid extraction for SARS-CoV-2 RNA testing. Negative control samples were supernatants obtained from A549 cells not infected with SARS-CoV-2.

SARS-CoV-2 immunofluorescence assay

The presence of SARS-CoV-2 antigens in A549 infected cells was evaluated by indirect immunofluorescence (IF) using polyclonal antibodies directed against N and S proteins of SARS-CoV-2(Sino Biological, Beijing, China). For the IF assay, the cells grown in 8-well chamber slide were rinsed twice with PBS, fixed in methanol solution at room temperature for 30 min, washed three times with PBS, and then incubated with the specific anti-SARS-CoV-2 antibodies (diluted 1:60 in PBS) for 1 hour at 37°C. After 3 washes with PBS, secondary antibody consisted of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (diluted 1:1000 in PBS; Sigma, St. Louis, MI, USA) was added for 1 hour at 37 °C.After three final washings, drying, and mounting, the slides were observed under a fluorescence microscope.

SARS-CoV-2 RNA detection

SARS-CoV-2 genome was detected by extracting RNA from 400 ml of cell supernatant using the Nimbus platform (Hamilton, Reno, NV, USA). After extraction, viral RNA was amplified by using the *Allplex*™ 2019-nCoVassay (Seegene, Seoul, South Korea) on the CFX96 instrument (Bio-Rad, Hercules, CA, USA), according to the manufacturer's instructions. The real-time *Allplex*™ 2019-nCoV assay simultaneously detects the N gene, the E gene, and the RdRP gene of the SARS-CoV-2 genome. The cycle threshold (Ct) value was recorded for each of the 3 genes. Samples were considered positive when a PCR signal was detected at C_t<40 for any gene.