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MAJOR ARTICLE

Effect of Ethanol Vapor Inhalation Treatment on Lethal Respiratory Viral Infection With Influenza A

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Ethanol (EtOH) effectively inactivates enveloped viruses in vitro, including influenza and severe acute respiratory syndrome coronavirus 2. Inhaled EtOH vapor may inhibit viral infection in mammalian respiratory tracts, but this has not yet been demonstrated. Here we report that unexpectedly low EtOH concentrations in solution, approximately 20% (vol/vol), rapidly inactivate influenza A virus (IAV) at mammalian body temperature and are not toxic to lung epithelial cells on apical exposure. Furthermore, brief exposure to 20% (vol/vol) EtOH decreases progeny virus production in IAV-infected cells. Using an EtOH vapor exposure system that is expected to expose murine respiratory tracts to 20% (vol/vol) EtOH solution by gas-liquid equilibrium, we demonstrate that brief EtOH vapor inhalation twice a day protects mice from lethal IAV respiratory infection by reducing viruses in the lungs without harmful side effects. Our data suggest that EtOH vapor inhalation may provide a versatile therapy against various respiratory viral infectious diseases.

Keywords. ethanol vapor; influenza; inhalation treatment; respiratory infection; enveloped viruses.

Respiratory viral infections are global health and economic risks that are difficult to control [1, 2], as illustrated by the coronavirus disease 2019 (COVID-19) pandemic [3, 4]. Effective vaccines can reduce the risk of specific respiratory infections in many people [5, 6], but additional therapeutic strategies should be developed to protect those who cannot access such vaccines. Furthermore, to address the risks of emerging mutant viruses that are resistant to vaccine-induced immune responses and new pandemic viruses in the future [7–10], therapeutic strategies applicable to a wide range of respiratory infectious diseases are needed. Inhalation of compounds that are cheap and that effectively target general viral properties or functions in the respiratory tract might be useful to control respiratory infections [11].

Ethanol (EtOH) is widely used for disinfection of environmental and body surfaces in daily life because it effectively inactivates bacteria and enveloped viruses [12]. For example, influenza A virus (IAV) and severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) are inactivated by exposure to about 30% EtOH in approximately 1 minute [13–15]. Theoretically, inhalation of EtOH vapor can expose the respiratory epithelium to enough EtOH to inactivate enveloped viruses [16]. Indeed, a molecular imaging study detected substantial amounts of EtOH in the lungs of rats following inhalation of EtOH vapor [17]. These observations support the therapeutic potential of EtOH vapor inhalation against respiratory infectious diseases, but there is no direct evidence that inhaled EtOH inhibits viral respiratory infections without damaging epithelial cells.

METHODS

Cells and Mice

Madin-Darby canine kidney (MDCK) cells (NBL-2; JCRB9029) were maintained in minimum essential medium (MEM; 11095080; Thermo Fisher Scientific) containing 10% fetal bovine serum (173012; Sigma-Aldrich). The human lung cancer cell line A549 (RCB0098) was provided by the RIKEN BioResource Research Center through the National Bio-Resource Project of the Ministry of Education, Culture, Sports, Science and Technology/Japan Agency for Medical Research and Development, Japan, and was maintained in Dulbecco's MEM (DMEM; D6429; Sigma-Aldrich) containing 10% fetal bovine serum. Female 6–8-week-old, specific pathogen-free C57BL/6J mice were obtained from Japan SLC. Protocols of all mouse experiments were approved by the Animal Care and Use Committee at the Okinawa Institute of Science and Technology Graduate University.

Influenza Virus

Influenza A/PR/8/34 virus (IAV; American Type Culture Collection, VR-95) was propagated in MDCK cells cultured in

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DMEM containing 0.35% bovine serum albumin (BSA; A7979; Sigma-Aldrich) and 0.12% sodium bicarbonate (199-05985; Wako). Medium was centrifuged at 2000*g* for 30 minutes at 4°C, followed by ultracentrifugation at 18 000*g* for 30 minutes at 4°C to remove cell debris. Supernatant was then layered on top of 5 mL of a chilled 5% sucrose cushion and ultracentrifuged at 112 000*g* for 90 minutes at 4°C to purify the virus. The viral pellet was resuspended in phosphate-buffered saline (PBS) and stored at −80°C. The titer of the viral stock was determined as described below.

IAV Titration

Titers of viral stock were measured by indirect immunofluorescence [18], while those of infectious viruses in other test samples were determined by median tissue culture infective dose ($TCID_{50}$) assay [19]. Briefly, confluent monolayers of MDCK cells cultured in 96-well tissue culture plates were infected with viral samples serially diluted 10-fold in infection medium (DMEM supplemented with 0.35% BSA and 0.12% sodium bicarbonate) for 1 hour at 37°C. The inoculum was then replaced with infection medium supplemented with 0.75 μg/mL tosyl phenylalanyl chloromethyl ketone–trypsin. In the indirect immunofluorescence assay, 8 hours after infection, cells were fixed, permeabilized, and blocked with 2.5% BSA (wt/vol) in PBS. Cells were reacted with 0.7 μg/mL anti-nucleoprotein (H16-L10-4R5) antibody (GTX14213; GeneTex) and then with Alexa Fluor Plus 555–conjugated anti-mouse immunoglobulin G secondary antibody (A32727, Thermo Fisher Scientific), and analyzed using fluorescence microscopy (BZ-X710; Keyence). In the $TCID_{50}$ assay, on day 4 after infection, cells were fixed with 4% paraformaldehyde in PBS and stained with crystal violet dye (C3886; Sigma-Aldrich). The $TCID_{50}$ was determined with the Reed–Muench method.

Cell Viability Assay

Cell viability was assessed with a Cell Counting Kit-8 assay (CCK-8; Dojindo laboratories), according to the manufacturer's instructions. Briefly, cells were incubated with CCK-8 solution for 1 hour at 37°C, and absorbance was measured at 450 nm using a microplate reader (iMark; Bio-Rad).

Transepithelial Electrical Resistance Assay

To evaluate the integrity of the epithelial monolayer culture, we measured transepithelial electrical resistance was measured using a Millicell ERS-2 (Millipore). During the measurement, cells on a cell culture insert in a tissue culture plate were incubated with PBS (300 μL in the apical compartment and 700 μL in the basolateral compartment) at 37°C. Relative transepithelial electrical resistance values were calculated based on the values before EtOH treatment.

EtOH Vapor Exposure System

A modified anesthesia induction chamber (MK-ICS; Muramachi) connected to an ultrasonic humidifier (TF003; Repti Zoo; flow rate, 26 L/min) was used as an exposure chamber, which allowed simultaneous exposure of up to 5 mice. In all animal experiments, 50% (vol/vol) EtOH solution preheated to 50°C was vaporized in high-power mode (atomizing amount when water was vaporized, 3.1 mL/min). As a control, distilled water preheated to 33°C was vaporized in medium-power mode (atomizing amount, 1.8 mL/min). In these conditions, about 30 mL of solvents were vaporized in 10 minutes, and the temperature in the exposure chamber was about 30°C.

Measurement of EtOH Concentrations

Concentrations of EtOH solutions were measured using a refractometer calibrated for EtOH (Yieryi; THE01507), according to the manufacturer's instructions. Briefly, 5–40-μL samples were analyzed, and standard curves were generated with known concentrations of EtOH diluted in distilled water or PBS. EtOH gas concentration was measured using an optical interferometer (FI-8000; Riken Instruments).

Henry's Law: Estimation of EtOH Concentration in Solution Exposed to EtOH gas

The amount of EtOH gas dissolved in a liquid is proportional to its partial pressure above the liquid, as follows:

$$C_a = K_{\mathrm{H}} \cdot p,$$

where C_a is a concentration of EtOH in solution, K_{H} is Henry's coefficient of EtOH for water, and p is the partial pressure of EtOH gas. At 25°C (absolute temperature $[T] = 298$ K), the average EtOH measured value is 194 mol/L/atmospheric pressure (atm; 1 atm = 101.3 kPa) [20]. The temperature dependence of the coefficient is calculated as follows:

$$d[ln(K_H)]/d(1/T) = 6274.$$

At 37°C ($T = 310$ K), we have 87.2 mol/L/atm. EtOH 4% (vol/vol) gas has a partial pressure of 4 kPa (0.04 atm), and the soluble EtOH in water becomes $87.2 \times 0.04 = 3.5$ mol/L, which is equal to 20.4% EtOH (vol/vol) in solution, where the molecular weight of EtOH is 46.1 g/mol, and the density is 0.79 g/mL.

EtOH Vapor Inhalation Treatment of Mice

A maximum of 5 mice were placed in the exposure chamber and exposed to EtOH vapor for 10 minutes twice a day. Mice were housed in cages between treatments and weighed daily.

Mouse Blood EtOH Measurement

Mouse blood EtOH concentration was assessed with an Alcohol Assay Kit (Colorimetric; STA-620; Cell Biolabs). Briefly, blood samples collected from tails were incubated at room temperature for 30 minutes, followed by centrifugation at 2500*g* for 20 minutes. Serum was collected and diluted in 1× assay buffer (1:50). Absorbance was measured at 570 nm using a microplate reader (iMark; Bio-Rad).

IAV Infection of Mice

Mice were anesthetized with isoflurane and intranasally injected with 1×10^3 fluorescence-forming units of IAV in 30 μL PBS. For viral titration, mice were euthanized on day 3 after infection, and their lungs and nasal mucosae were isolated and homogenized using a PowerMasher II homogenizer (891300; Nippi) at room temperature, stored at −80°C, and subjected to $TCID_{50}$ titration assays.

Histopathological Analysis

Lung samples were fixed in 4% paraformaldehyde phosphate buffer. Sectioning and hematoxylin-eosin staining were performed with GenoStaff. Hematoxylin-eosin–stained sections of the lung were analyzed with an all-in-one fluorescence microscope (BZ-X710; Keyence).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (version 9.4.0), and statistical details are provided in the figure legends.

RESULTS

Inactivation of IAV by Ethanol Solution at Room Temperature

We reasoned that inhalation of EtOH vapor protects the host from respiratory viral infection if the EtOH concentration in the respiratory epithelial lining fluid is sufficient to inactivate the viruses without being toxic to epithelial cells. Because in vivo virucidal activity of EtOH remains to be determined, we first sought to address this by exposing IAV to various concentrations of aqueous EtOH solution at body temperature (37°C) or room temperature (24°C) for 1 minute. Consistent with previous reports [14], the lowest concentration of EtOH solution that completely inactivated IAV at 24°C was 36% (vol/vol) (Figure 1*A*). In contrast, surprisingly, 22.5% (vol/vol) EtOH solution effectively inactivated IAV to undetectable levels at 37°C (Figure 1*B*). Furthermore, even 18% (vol/vol) EtOH solution reduced infectious IAV >10-fold at 37°C (Figure 1*B*). In the absence of EtOH, the infectivity of IAV decreased in a temperature-dependent manner above 55°C, reaching undetectable levels at 61°C (Figure 1*C*). Minimal EtOH concentrations required to inactivate IAV to undetectable levels were inversely proportional to the reaction temperature (Figure 1*D*), suggesting that thermal and chemical energies contribute cooperatively to viral inactivation. Taken together, these data suggest that an approximately 20% (vol/vol) EtOH solution might be sufficient to inactivate IAV in the respiratory tract.

Effect of Apical Exposure of Lung Epithelial Cells to EtOH on Cell Viability and Progeny Virus Production

Next, we investigated susceptibility of respiratory epithelial cells to EtOH. When compounds are inhaled, respiratory epithelial cells are exposed from the apical side, which is covered by the epithelial lining fluid in the lumen of respiratory tracts [21]. To assess cytotoxic effects of apical EtOH exposure, we exposed A549 cells to 22.5% (vol/vol) EtOH from the apical side (Figure 2*A*). The concentration of EtOH solution in the apical fluid was slightly decreased in 10 minutes, while that in the basolateral compartment was undetectable (Figure 2*B*). Notably, apical exposure to 22.5% (vol/vol) EtOH solution for 10 minutes did not affect A549 cell viability (Figure 2*C*). However, there was a significant reduction in cell viability on exposure to 22.5% EtOH from both the apical and basolateral sides (Figure 2*C*) or apical exposure to concentrations of EtOH >27.0% (vol/vol) (Supplementary Figure 1*A*).

To investigate whether EtOH exposure inhibits progeny virus production in IAV-infected cells, we treated IAV-infected A549 cells with apical exposure to 22.5% (vol/vol) EtOH for 1 minute at 6 hours after infection. Interestingly, EtOH significantly reduced viral production over the course of 18 hours after treatment (Figure 2*D*) without affecting cell viability (Supplementary Figure 1*B*). Antiviral innate immunity inhibits intracellular IAV infection process [22], but we observed that EtOH treatment did not affect infection-induced expression of genes critical for innate immunity, such as the gene for interferon regulatory factor 7 (*IRF7*) [23–25] (Supplementary Figure 1*C*). These results suggest that respiratory epithelial cells are tolerant to brief apical exposure to EtOH solution up to about 20% (vol/vol) and that EtOH inhibits intracellular IAV infection process.

Generation of EtOH Vapor as an In Vivo Virucide

Based on our in vitro results, we hypothesized that inhaled EtOH vapor would inactivate enveloped viruses in respiratory epithelial lining fluid without cytotoxicity by increasing EtOH concentration in the fluid to about 20% (vol/vol). To verify our hypothesis, we developed an EtOH exposure system that can simultaneously expose up to 5 mice to EtOH vapor generated from an ultrasonic humidifier (Figure 3*A*). From Henry's law, it was estimated that exposure of solvent to 4% (vol/vol) EtOH gas would result in generation of about 20% (vol/vol) EtOH solution at 37°C by gas-liquid equilibrium. We first tested whether the EtOH exposure system can generate EtOH gas concentrations of 4% (vol/vol) or higher. Because EtOH solutions at concentrations of ≥67% (vol/vol) are considered hazardous under the Japanese Fire Service Law, we decided to use 50% (vol/vol) EtOH solutions as a vapor source. We observed that vaporization of 50% (vol/vol) EtOH solution preheated to 50°C was sufficient to generate 4% (vol/vol) EtOH gas (Figure 3*B*). Furthermore, exposure to the EtOH gas generated in this condition increased EtOH concentrations in the apical fluid of A549 cell culture to >20% (vol/vol) within 6 minutes (Figure 3*C*). These results suggest that an optimized EtOH vaporizing protocol can generate enough EtOH gas to increase the EtOH concentration in respiratory epithelial lining fluid to

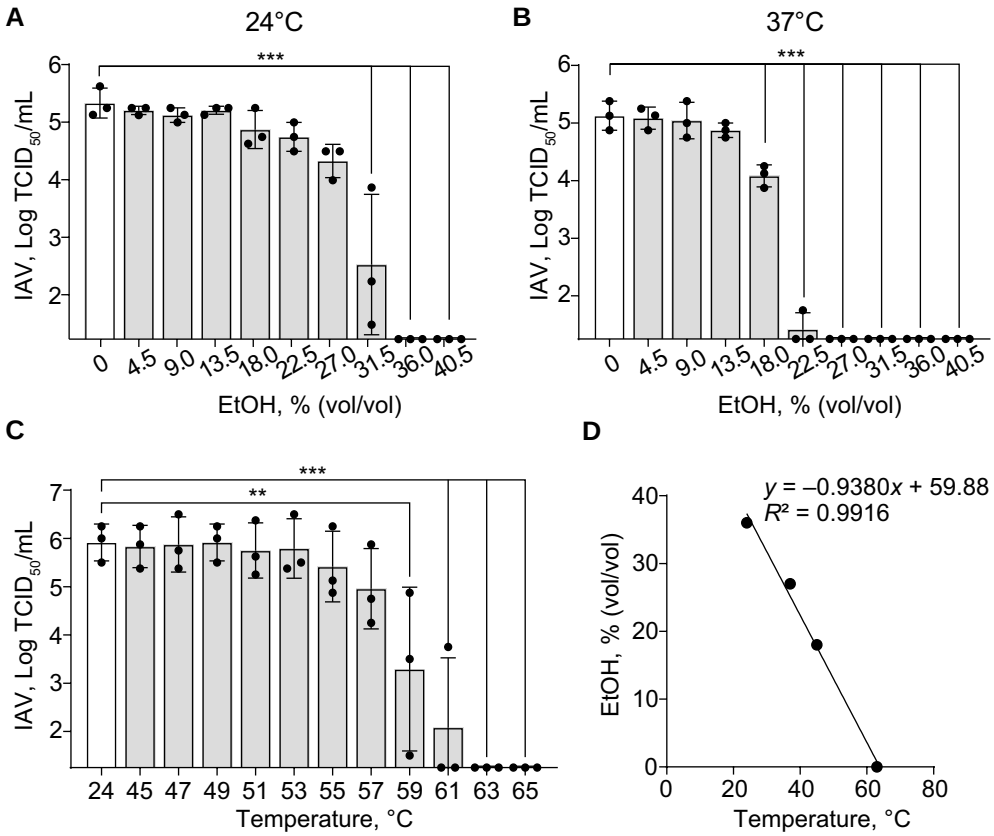

Figure 1. Virucidal efficacy of ethanol (EtOH) depends on temperature. *A–C*, Influenza A virus (IAV) suspended in 50 μL of phosphate-buffered saline (PBS) was mixed with 450 μL of PBS containing 0%–45% (vol/vol) EtOH preheated to reaction temperatures and incubated for 1 minute at 24°C (*A*), 37°C (*B*), or various temperatures (*C*). Final concentrations of EtOH and reaction temperatures are indicated in each panel. The reaction was terminated by adding 9 volumes of Dulbecco's minimum essential medium supplemented with 0.35% bovine serum albumin, 0.12% sodium bicarbonate, 1% antibiotic-antimycotic, and 0.75 μg/mL tosyl phenylalanyl chloromethyl ketone–trypsin–trypsin, and viral titers were determined. Data are expressed as means with standard deviations (n = 3). $^{**}P < .01$; $^{***}P < .001$ (calculated using 1-way analysis of variance with Dunnett multiple comparison tests). Abbreviation: $TCID_{50}$, median tissue culture infective dose. *D*, Scatterplot showing an inverse linear relationship between EtOH concentrations required for inactivation of IAV and reaction temperature. Data are representative of ≥2 independent experiments.

about 20% (vol/vol), a level sufficient to inactivate IAV at body temperature.

Safety of EtOH Vapor Inhalation

To evaluate the safety of EtOH vapor inhalation treatment, we exposed mice to EtOH vapor generated by vaporizing 50% (vol/vol) EtOH solution preheated to 50°C for 10 minutes twice a day. We observed that mice walked unsteadily after a brief (10-minute) exposure to EtOH vapor but recovered to normal within 20 minutes (data not shown). Blood alcohol concentrations (BACs) were about 0.052% (0.019%) (mean [standard deviation (SD)]) (wt/vol) at 15 minutes after EtOH vapor inhalation treatment but decreased to undetectable levels in 5 hours (Figure 4*A* and Supplementary Figure 2*A*). We also detected a mean (SD) of 0.024 (0.016) mg of EtOH in the bronchoalveolar lavage fluid (BALF) immediately after a brief EtOH vapor exposure (Supplementary Figure 2*B*). Assuming that the volume of the epithelial lining fluids in murine lungs is 80 μL (thickness of the fluid, 0.1 μm; surface area of the lungs, 800 cm^2), the mean (SD) concentrations of EtOH in the lung epithelial lining fluid was estimated to be 0.030% (0.020%) (wt/vol). Brief EtOH vapor exposure twice a day did not alter body weight or serum levels of aspartate aminotransferase and alanine aminotransferase until 3 weeks of treatment (Figure 4*B* and 4*C*). Moreover, EtOH vapor treatment also did not cause detectable tissue damage or inflammation in the nasal cavity and lungs, nor did it result in hepatic steatosis (Figure 4*D* and Supplementary Figure 2*C* and 2*D*). These results indicate that short-term use of daily brief inhalation of EtOH vapor does not have adverse effects on mice.

Protection of Mice from Lethal IAV Infection by EtOH Vapor Inhalation

Finally, we assessed the in vivo virucidal activity of EtOH vapor inhalation treatment using a mouse respiratory infection model of IAV. Mice were treated with EtOH vapor twice a day from the day before intranasal administration of IAV (1×10^3 fluorescence-forming units per mouse). Mice treated with EtOH vapor exhibited less weight loss than control mice

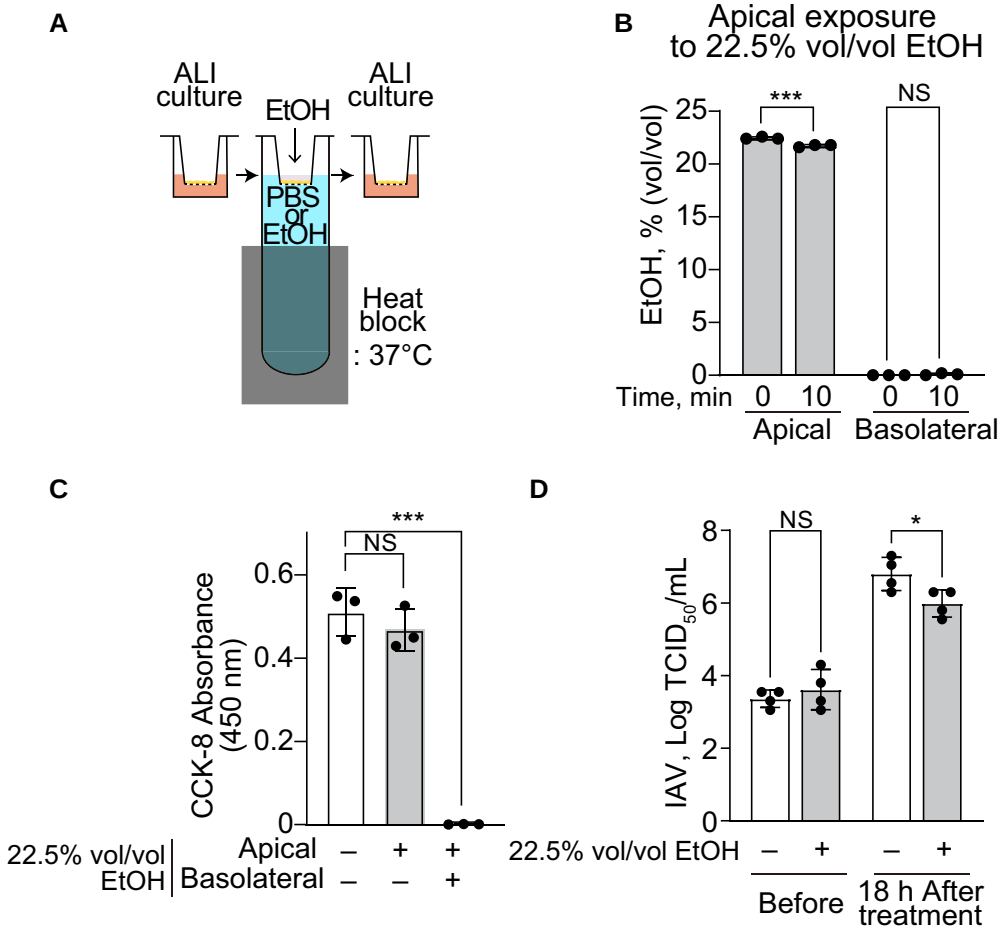

Figure 2. Resistance of lung epithelial cells to apical exposure to ethanol (EtOH). *A*, Schematic representation of experimental settings. A549 cells (3×10^4) seeded on a cell culture insert in a 24-well tissue culture plate were grown under air-liquid interface (ALI) culture conditions in which basolateral surfaces of the cells were in contact with culture medium. Culture medium was changed every 2–3 days, and cells were cultured for ≥25 days until EtOH exposure. Cell numbers and integrity of epithelial monolayers were assessed with a Cell Counting Kit-8 (CCK-8) and transepithelial electrical resistance (TEER) assays, respectively, and cells with CCK-8 values >0.5 and TEER values >20 were used for EtOH exposure assays. After washing cells on the cell culture insert with phosphate-buffered saline (PBS), the insert was placed on a tube (352059; Falcon) containing 12.25 mL of PBS with (for exposure from both the apical and basolateral sides) or without various concentrations of EtOH (for exposure from the apical side only), and 500 μL of PBS containing EtOH was added to the apical compartment of the insert. *B*, EtOH concentrations in apical and basolateral compartments were measured after 10 minutes of exposure of cells to 22.5% EtOH from the apical side only (n = 3). *C*, Analysis of the viability of cells unexposed (indicated by minus symbols) or exposed to 22.5% EtOH from the apical side only or from both the apical and basolateral sides (indicated by plus symbols). After 10-minute exposure, cells were washed with PBS and cultured under ALI conditions for 24 hours. Cell viability was assessed with a CCK-8 assay (n = 3). *D*, Analysis of progeny virus production in cells infected with influenza A virus (IAV) and then unexposed (indicated by minus symbols) or exposed (indicated by plus symbols) to EtOH. A549 cells were infected with IAV at a multiplicity of infection of 1. At 6 hours after infection, cells were apically exposed to 22.5% (vol/vol) EtOH for 1 minute. After washing with PBS, cells were cultured for 18 hours, and viral titers in culture medium were measured (n = 4). Abbreviation: $TCID_{50}$, median tissue culture infective dose. *B–D*, Data are expressed as means with standard deviations and are representative of ≥2 independent experiments. $^*P < .05$; $^{***}P < .001$; NS, not significant (calculated using 1-way analysis of variance with Dunnett multiple comparison tests).

(Figure 5*A*). By day 10 after infection, 63% (17 of 27) of the untreated mice reached the humane end point (20% loss of original body weight), compared with only 11% (3 of 27) of the mice treated with EtOH vapor (Figure 5*B*). In our EtOH exposure system, mouse bodies are exposed to EtOH vapor, which may lead to oral ingestion of EtOH adhering to the fur. To rule out the possibility that ingested EtOH inhibits lethal IAV infection, we applied a 22.5% EtOH solution to the body surface of mice. Application of EtOH to the body surface did not increase BAC as high as EtOH vapor inhalation treatment, nor did it inhibit lethal IAV infection (Supplementary Figure 3*A* and 3*B*).

Notably, EtOH vapor treatment decreased viral titers in the lungs, but not in the nasal cavity, on day 3 after infection (Figure 5*C*). EtOH vapor treatment also reduced leukocyte infiltration and damage in the lungs (Figure 5*D* and Supplementary Figure 4*A*). Consistent with this, EtOH vapor treatment decreased numbers of monocytes and macrophages in BALF (Supplementary Figure 4*B–D*), although it tended to increase the viability of these cells (Supplementary

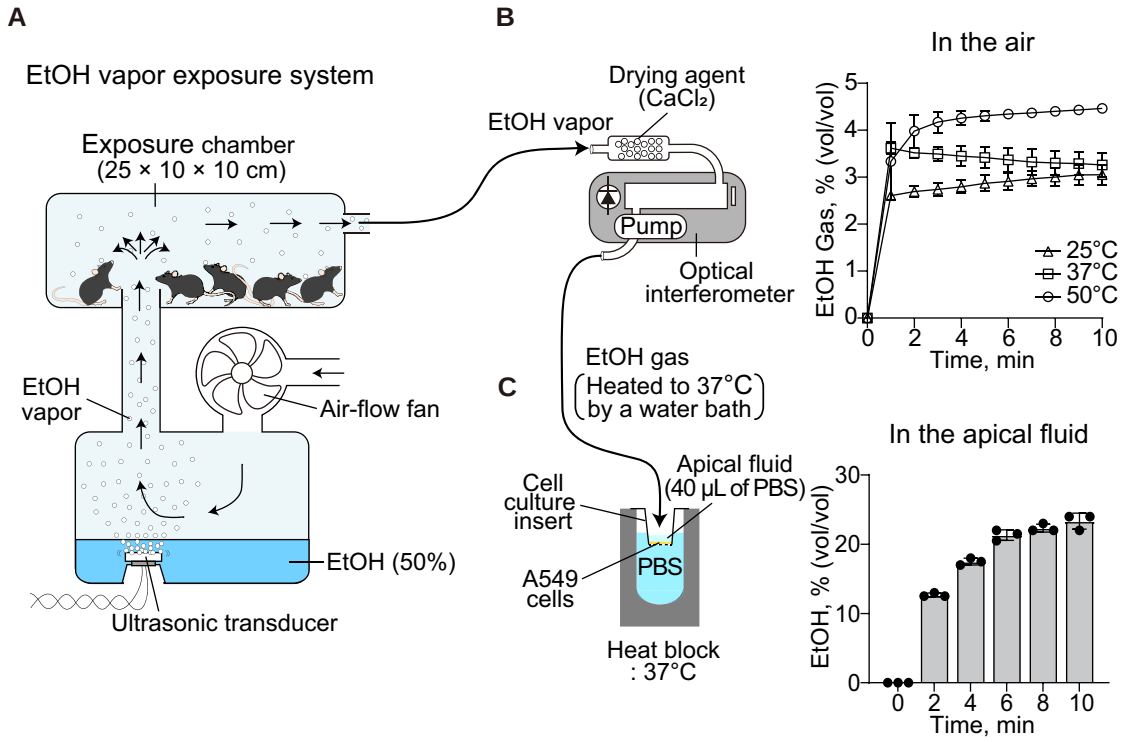

Figure 3. Ethanol (EtOH) vapor-mediated gas-liquid equilibrium. *A*, Schematic diagram of the EtOH vapor exposure system. EtOH solutions are vaporized in an ultrasonic humidifier, and vaporized EtOH flows into the exposure chamber for mice and is released from the air outlet. Dimensions of the chamber represent width × depth × height. *B*, Analysis of concentrations of EtOH gas generated by the EtOH vapor exposure system; 50% EtOH solutions preheated to 25°C, 37°C, or 50°C were vaporized in the EtOH exposure system, as shown in *A*. The concentration of EtOH gas released from the exposure chamber was analyzed by an optical interferometer connected via a tube containing calcium chloride ($CaCl_2$) as a desiccant. Schematic diagram on left shows the experimental design; graph right, the change in EtOH gas concentrations (vol/vol in the air) for 10 minutes after EtOH vaporization. *C*, Analysis of EtOH concentrations in the apical fluid of A549 cells exposed to EtOH gas at 37°C. EtOH gas produced by vaporizing a 50% EtOH solution preheated to 50°C in the EtOH vapor exposure system was released on the apical fluid (40 µL of phosphate-buffered saline [PBS]) of A549 cells cultured on a cell culture insert placed in PBS at 37°C, via the pump of the optical interferometer shown in *B*. Schematic diagram on left shows the experimental design; graph on right, changes in EtOH concentration (vol/vol) in the apical fluid. *B, C*, Data are expressed as means with standard deviations (n = 3) and are representative of 2 independent experiments.

Figure 4*E*). Furthermore, EtOH vapor treatment significantly decreased expression of IAV-induced genes associated with innate immunity, such as *Irf7*, in lung tissues (Supplementary Figure 4*F*). These results indicate that EtOH vapor inhalation reduces viral load in the lungs and protects mice from lethal IAV infection.

DISCUSSION

The current study demonstrates that short-term use of EtOH vapor inhalation treatment, initiated before infection, is safe and effectively protects mice from lethal respiratory infection with IAV. Brief EtOH vapor inhalation treatment twice a day significantly reduced viral titers in the lungs and ameliorated lung damage caused by IAV infection. On the other hand, when healthy mice were treated with brief EtOH vapor inhalation twice a day for 3 weeks, there were no detectable adverse effects. These results confirm the safety of short-term use of EtOH vapor inhalation treatment and provide evidence of EtOH's virucidal activity in vivo.

Our results suggest that the virucidal efficacy of low concentrations of EtOH solution for the respiratory tract is higher than that for environmental surfaces, owing to temperature effects. Inactivation of IAV at room temperature requires EtOH solutions more concentrated than 30% (vol/vol) [13–15]. However, at body temperature, IAV is inactivated within 1 minute after exposure to 22.5% (vol/vol) EtOH solution. Interestingly, apical exposure of IAV-infected cells to 22.5% (vol/vol) EtOH for 1 minute significantly reduced progeny virus yield over the course of 18 hours after exposure. Given these observations, we speculated that, if EtOH vapor inhalation increases concentrations of EtOH in the respiratory epithelial lining fluids to about 20%, EtOH not only directly inactivates extracellular IAV but also suppresses progeny virus production in the respiratory tract. Inactivation of extracellular IAV by EtOH is likely due to its ability to disrupt viral envelope [26, 27], but how

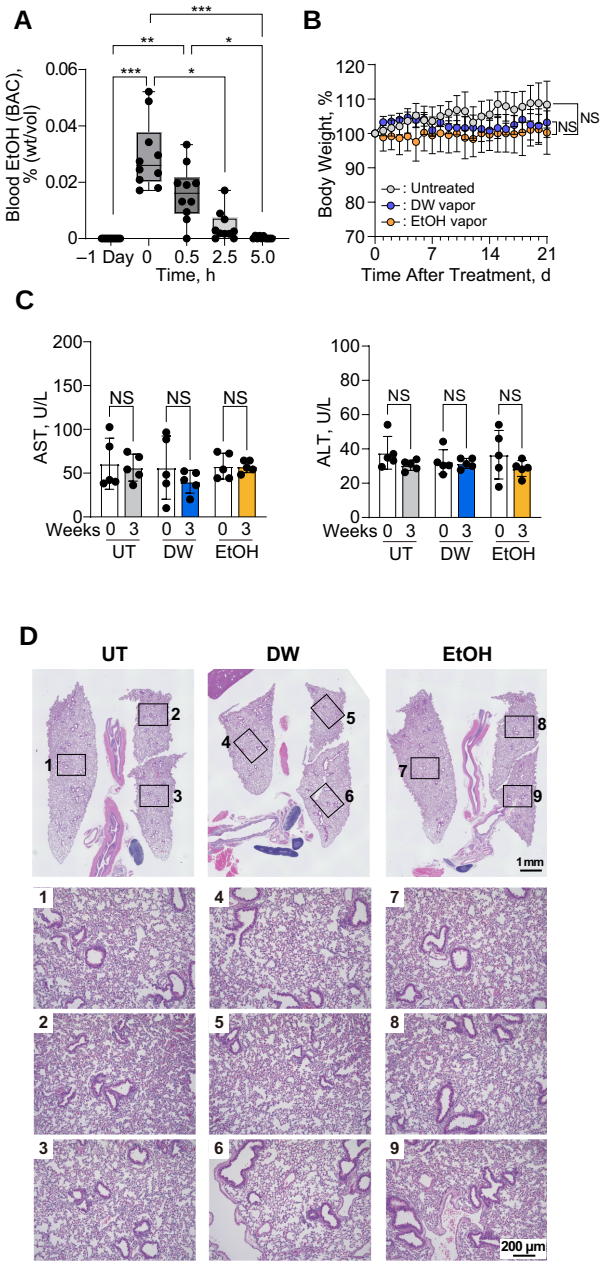

Figure 4. Ethanol (EtOH) vapor inhalation treatment does not damage bronchial tissues. Female C57Bl/6 mice were placed in the exposure chamber and exposed to EtOH or distilled water (DW) vapor for 10 minutes every 12 hours for 3 weeks. *A,* Blood EtOH concentration (blood alcohol concentration [BAC]) was measured after EtOH vapor inhalation. $^{*}P < .05$; $^{**}P < .01$; $^{***}P < .001$ (calculated using 1-way analysis of variance [ANOVA] with Tukey's multiple comparison tests). *B,* Body weight changes in mice treated with EtOH or DW vapor inhalation or untreated (UT) (n = 5). $^{*}P < .05$; NS, not significant (calculated using 2-way ANOVA with Tukey's multiple comparison tests). *C,* Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were measured (n = 5); *P* values were calculated using unpaired *t* tests followed by correction using Benjamini-Hochberg false discovery rate for multiple comparisons. *D,* Histopathological analysis of lungs isolated from mice treated without or with DW or EtOH vapor inhalation on day 21. Enlarged images of areas shown in open boxes in the top panels are shown in panels 1–9. All data in *A–D* are representative of ≥2 independent experiments.

EtOH regulates progeny virus production is enigmatic. Although we observed that IAV-induced expression of type I interferon-related genes was not affected by EtOH, further studies are needed to determine whether EtOH enhances antiviral immunity, because EtOH modulates several immune-related pathways [28–33]. Given previously reported EtOH activity [34–36], it is also important to evaluate the effect of EtOH on activity of viral proteins or cellular metabolism in virus-infected epithelial cells.

We used an EtOH exposure system that can generate about 4% (vol/vol) EtOH gas for EtOH inhalation treatment of mice. Although the lower explosion limit (LEL) of EtOH gas is 3.5% (vol/vol) in dry conditions, in our preliminary experiments, 4% (vol/vol) EtOH gas generated by vaporization of 50% (vol/vol) EtOH solution did not explode on ignition, probably owing to abundant water vapor. Consistent with estimation from Henry's law, we observed that on exposure to 4% (vol/vol) EtOH gas, about 20% (vol/vol) EtOH solution was generated in the apical compartment of lung epithelial cell culture at 37°C. This supports the hypothesis that EtOH vapor inhalation protects mice from lethal IAV infection by raising the concentrations of EtOH in the respiratory epithelial lining fluids to 20% (vol/vol). However, it is challenging to demonstrate this in vivo since it is impossible to directly measure EtOH concentrations in the respiratory tract with current technology [37]. Based on our data, the mean (SD) concentration of EtOH in the lung epithelial lining fluid was estimated to be 0.030% (0.020%) (wt/vol), the same range as for BAC, in mice treated with EtOH vapor. As observed with other compounds [37], EtOH may be rapidly absorbed from the lungs into the systemic circulation during a few minutes of BALF collection. EtOH pharmacokinetics in the lung should be further studied using computational fluid dynamics simulation.

Our data support the safety of brief EtOH vapor inhalation. Although cytotoxic effects of EtOH were previously observed in various cells [38], we found that respiratory epithelial cells are tolerant to exposure of their apical sides to 20% (vol/vol) EtOH solution, as long as their basolateral sides are not exposed to such concentrations. Consistent with this, brief EtOH vapor inhalation twice a day did not cause body weight changes, damage in the nasal cavity and lungs, hepatic steatosis, or elevation of serum alanine aminotransferase and aspartate aminotransferase levels until 3 weeks of treatment. In mice treated with EtOH vapor, BAC increased up to about 0.05% (wt/vol) and decreased rapidly in a few hours. Assuming a similar effect in humans, EtOH vapor inhalation may transiently raise BAC to the Japanese legal limit for motor vehicle operation (0.03% [wt/vol]), but not to the United States legal limit (0.08% [wt/vol]). These results suggest that short-term use of brief EtOH vapor inhalation treatment does not likely cause adverse side

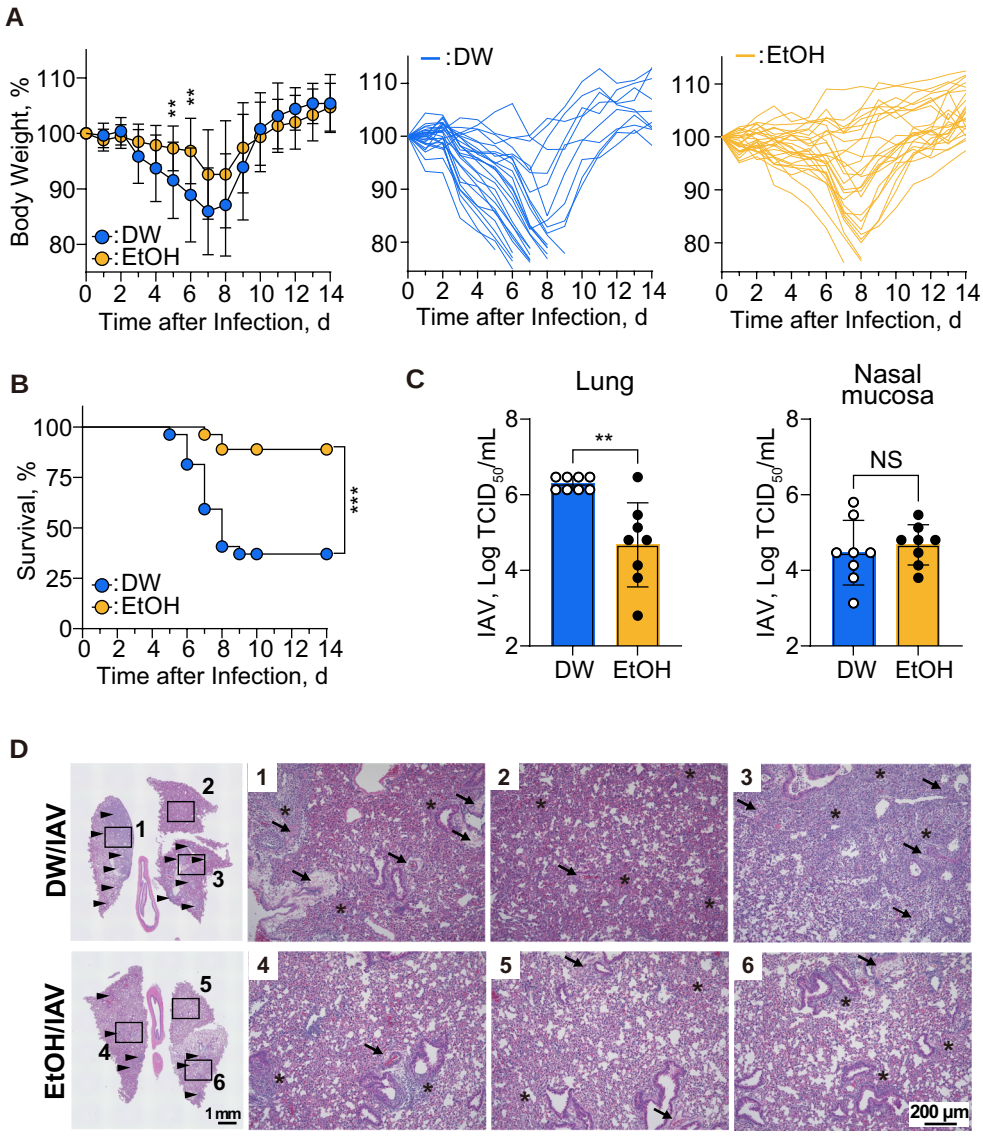

Figure 5. Ethanol (EtOH) vapor inhalation treatment suppresses pulmonary infection with influenza A virus (IAV). From 1 day before the IAV infection (day −1), mice were subjected to distilled water (DW) or EtOH vapor inhalation treatment for 10 minutes twice a day throughout the experiment, as in Figure 4. On day 0, mice were intranasally injected with 1×10^3 fluorescence-forming units of IAV. On the same day, EtOH vapor inhalation treatment was performed 4–6 hours before and after infection. *A*, *B*, Body weight changes (*A*) and survival rates (*B*) in mice treated with DW or EtOH vapor inhalation. Mice were weighed daily, and those showing 20% weight loss were considered to be dying and were humanely euthanized. Data are pooled from 4 independent experiments (n = 27). *A*, Left graph shows means with standard deviations (SDs) of weights in each group; middle and right graphs, weights of individual mice treated with DW vapor or EtOH vapor, respectively. (** $P < .01$ (calculated with multiple Mann-Whitney tests). *B*, Kaplan-Meier survival curves were calculated. ** $P < .01$; *** $P < .001$ (calculated with log rank test). *C*, Viral titers in the lungs and nasal cavity on day 3. Data are pooled from 2 independent experiments and expressed as means with SDs (n = 8). ** $P < .01$; NS, not significant (calculated using unpaired *t* tests). Abbreviation: $TCID_{50}$, median tissue culture infective dose. *D*, Histopathological analysis of lungs isolated from mice treated without or with EtOH vapor inhalation on day 3. IAV-induced lung inflammation is characterized by leukocytic infiltration (*asterisks*), alveolar collapse (*arrows*), and focal inflammation in the lung (*arrowheads*). Zoomed-in images of areas indicated in open boxes in the left panels are shown in panels 1–6. Representative results are shown for 1 mouse of 5 in each group (others shown in Supplementary Figure 4*A*).

effects, including alcohol-related chronic diseases, although the systemic effects of long-term usage need to be evaluated.

Our data suggest promising therapeutic potential of EtOH vapor inhalation against respiratory viral infection, but the virucidal efficacy of the current treatment protocol is still limited. EtOH vapor treatment reduced viral load 10-fold in the lung but not in the nasal cavities of IAV-infected mice. Because the surface-to-volume ratio of the epithelial lining fluids in the nasal cavity is much lower than that in the lung, where the thin surfactant covers a vast respiratory surface [21], brief exposure to 4% (vol/vol) EtOH gas might not be enough to saturate the nasal cavity fluids with EtOH. In addition, since temperatures in the nasal cavity are lower than in the lungs [39], minimum concentrations of EtOH required for IAV inactivation

are likely higher in the nasal cavity than in the lungs. It is worth evaluating whether the virucidal activity of EtOH vapor treatment is enhanced by increasing the treatment time or generating higher concentrations of EtOH gas.

In conclusion, EtOH vapor inhalation optimized to expose the respiratory tract to about 4% (vol/vol) EtOH gas can limit lethal IAV respiratory infection without damaging epithelial cells. Because various enveloped viruses, including SARS-CoV-2, are more susceptible to EtOH than influenza viruses [14], EtOH vapor inhalation treatment may be effective against respiratory infection of these viruses, which enter through and bud from the apical surface of the respiratory epithelium [40, 41]. Future studies should evaluate the therapeutic effects of EtOH vapor inhalation against various respiratory infectious diseases, including SARS-CoV-2 and avian IAV.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

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Author contributions. M. T. performed most of the experiments using mouse and cell line models. S. T. and M. Y. conducted the in vitro influenza A virus inactivation assay. T. M. assisted with experiments of ethanol vapor inhalation treatment of mice. H. I. and T. S. conceived, designed, and supervised the study. M. T., M. Y., H. I., and T. S. wrote the manuscript.

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