Inactivation of Mycobacteria by Radicals from Non-Thermal Plasma Jet^S

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Mycobacterial cell walls comprise thick and diverse lipids and glycolipids that act as a permeability barrier to antibiotics or other chemical agents. The use of OH radicals from a non-thermal plasma jet (NTPJ) for the inactivation of mycobacteria in aqueous solution was adopted as a novel approach. Addition of water vapor in a nitrogen plasma jet generated OH radicals, which converted to hydrogen peroxide (H₂O₂) that inactivated non-pathogenic *Mycobacterium smegmatis* and pathogenic *Mycobacterium tuberculosis* H37Rv. A stable plasma plume was obtained from a nitrogen plasma jet with 1.91 W of power, killing *Escherichia coli* and mycobacteria effectively, whereas addition of catalase decreased the effects of the former. Mycobacteria were more resistant than *E. coli* to NTPJ treatment. Plasma treatment enhanced intracellular ROS production and upregulation of genes related to ROS stress responses (thiol-related oxidoreductases, such as SseA and DoxX, and ferric uptake regulator *furA*). Morphological changes of *M. smegmatis* and *M. tuberculosis* H37Rv were observed after 5 min treatment with N₂+H₂O plasma, but not of pre-incubated sample with catalase. This finding indicates that the bactericidal efficacy of NTPJ is related to the toxicity of OH and H₂O₂ radicals in cells. Therefore, our study suggests that NTPJ treatment may effectively control pulmonary infections caused by *M. tuberculosis* and nontuberculous mycobacteria (NTM) such as *M. avium* or *M. abscessus* in water.

Keywords: Non-thermal plasma, hydroxyl radical, ROS stress, *Mycobacteria*

Introduction

Mycobacterium tuberculosis (Mtb) is an obligate human pathogen that causes tuberculosis. Nontuberculous mycobacteria (NTM), including *M. avium*, *M. kansasii*, and *M. abscessus*, are opportunistic pathogens that are frequently isolated from diverse environments such as water, soil, and tap water [1]. It is important to inactivate these pathogens present in the environment for prevention of the chronic pulmonary diseases which they cause. In both, gram-negative and gram-positive cells, a common mechanism of cellular death inflicted by bactericidal antibiotics is by the generation of OH radicals via Fenton's reaction [2]. It has been reported that rifampin-induced hydroxyl radicals participate at least partially, in killing Mtb [3], and that Mtb

is extraordinarily susceptible to vitamin C-mediated OH radicals [4].

The ability of OH radicals to induce biological damage is often exploited in the medical fields as therapeutic tools. Among these, reactive oxygen species (ROS) generated by non-thermal atmospheric-pressure plasma are an important means of inactivating microbial pathogens [5, 6]. More recently, the efficiency of the cold argon plasma jet and dielectric barrier discharge to inactivate mycobacteria were reported [7, 8]. They determined the size of growth inhibition in the solid medium and reported that the dielectric barrier discharge was an effective method. There is no study on oxidative stress by radicals in nitrogen plasma jet with *mycobacteria* in media. Atmospheric-pressure plasma jets produce short-lived reactive species,

such as OH radicals, including atomic oxygen and nitric oxide [9, 10]. These devices are compact and structurally flexible with low discharge power, and have been applied in biological and medical fields [11–13]. Such devices have been employed to inactivate *Staphylococcus*, *Streptococcus*, and even bacterial endospores [14–16]. However, the mycobacterial cell wall is much thicker than that in other gram-negative bacteria, and it also differs from other bacterial cells in biochemical composition [17]. In order to exert antimicrobial effects on Mtb by NTPJ, the plasma condition should be more efficient [9, 10, 18]. Production of OH radicals and hydrogen peroxide through our NTPJ device should be at low plasma dose, or enough to kill or inactivate bacteria. Control of this plasma dose can be a critical requirement for the development of an efficient plasma sterilizer [18].

We investigated the inactivating effect of plasma jets with N_2 gas alone, or a gas-mixture composed of N_2 and distilled water vapor on mycobacteria in aqueous solution. We found that the mycobacteria were more resistant than *E. coli* to NTPJ. The NTPJ with N_2+H_2O gas was more effective in generating the radicals and inactivating mycobacteria compared to N_2 gas alone. Plasma treatment induced intracellular ROS production and enhanced the expression of genes related to ROS in mycobacteria. Catalase significantly inhibited the responses generated by the plasma. The hydroxyl radicals generated by NTPJ are converted to hydrogen peroxide, which leads to antimicrobial effects, eventually resulting in gradual death of bacteria.

Materials and Methods

Non-Thermal Plasma Jet (NTPJ) Device

A discharge power at frequency, $f = 60$ Hz obtained from a high-voltage AC power supply, was passed through an electrode, and eventually fed into the plasma jet source (Fig. 1A). The glove box was vacuumed to eliminate any involvement of oxygen, and purged with pure nitrogen gas before the experiment. To generate plasma, N_2 gas alone or a mixture of N_2 and distilled water vapor, was introduced through a mass controller (MFC, ATOVAC1200). This device regulated the flow rate of N_2 gas at 600 cc/min (when N_2 gas alone was passed) and a flow rate of 400 cc/min, when N_2 gas was passed through the water-bottle vapor gas. The plasma jet source used in this study had a needle structure (Fig. 1B) made of stainless steel (1.3 mm in inner diameter and 0.3 mm thickness). Plasma was generated in the porous alumina-based ceramics between the electrodes, which were separated by a gap of 1 mm [19]. Nitrogen was injected into the electrode and ejected through the 1-mm hole in the outer electrode via the porous layers. The input power led to the generation of a large number of pulses

Fig. 1. (Color online) Non-thermal plasma jet device with bubbler (A) Schematic view of a non-thermal plasma generation system, (B) Schematic view (left panel), and picture (right panel) of nitrogen-plasma jet source, and (C) Voltage and current waveforms of the nitrogen-plasma jet, $V_{rms} = 2.15$ kV and $I_m = 0.89$ mA.

(Fig. 1C), accompanied by a subsequent decrease in V_{rms} to 2.15 kV (TDS2002C, Tektronix), and an increase in current, I_m , to 0.89 mA (P6021, Tektronix), corresponding to a 1-slm (standard liter per min) plasma jet (inset photograph, Fig. 1B) in the N_2+H_2O gas mixture. Optical spectroscopy of the discharge was performed using a spectrometer (Ocean Optics, HR4000, USA) at 0.7 nm resolution. The detailed design and function of NTPJ has been previously reported [19].

Bacteria Strains, Culture Conditions and Colony Assay

The slowly growing *Mycobacterium tuberculosis* H37Rv (ATCC

27294) and rapidly growing *Mycobacterium smegmatis* (Msm, ATCC 700084) were purchased from American Type Culture Collection (ATCC, Manassas, VA). The mycobacteria were grown at 37°C in 7H9 (BD Difco) supplemented with 10% OADC (Albumin bovine serum, vol/vol) and 0.2% glycerol. *Escherichia coli* (NCCP 14762) were cultured in Luria Bertani Broth until they reached the logarithmic growth phase. The cultured bacteria were diluted to 1×10^5 cells/ml and 1×10^7 cells/ml and 0.5 ml of the diluted bacterial solution was added to a 24-well plate (30024, SPL), and treated with the plasma jet. The bacteria after plasma exposure were serially diluted 10-fold in DPBS (LB001-02, Welgene) media, and colony assay was performed on 7H10 agar plates.

pH, Temperature and Radical Concentrations

Around 0.5 ml of 7H9 in a 24-well plate was treated with an NTPJ with N₂ alone and with a mixture of water and N₂ for 1, 3, and 5 min. The pH and radical concentration of each sample were measured immediately after plasma exposure. The pH was measured using a pH meter (Orion Star A211, Thermo Scientific) and the concentrations of hydrogen peroxide and nitrite in the 7H9 media were analyzed using a Quantichrom Peroxide Assay Kit (DIOX-250, BioAssay Systems) and Griess reagent, respectively. In order to verify the decomposition of H₂O₂, catalase, which is used to scavenge the hydrogen peroxide, was used. The cells were pre-incubated with the catalase for 15 min at room temperature and then treated with plasma. From 300 kU/ml of catalase solution (C3556, Sigma-Aldrich), 1.5 kU/ml was used. OH radicals were also measured using terephthalic acid (TA, Sigma-Aldrich). Ten millimolars of TA was prepared in 5 mM NaOH. Five millimolars of TA was added to 7H9 media, and fluorescence was measured at an excitation/emission of 310/425 nm (Synergy H1, Biotek) [20]. For temperature measurement, plasma was exposed to the center of the 7H9 well plate, maintaining a gap of 5 mm from the plasma source end. Immediately after plasma exposure, the center of the well plate was measured using FLIR (BCAM SD infrared thermal imager).

LIVE/DEAD BacLight Bacterial Viability Assay

Viability was determined using the BacLight Bacterial Viability

Kit (L7012, Thermo Fisher, USA) according to the manufacturer's protocol. Briefly, 3 µl of live/dead staining solution was added to 997 µl of cell suspension. The stained bacteria were then analyzed by confocal laser scanning microscopy (TCS SP8, Leica, Germany). Images of the mycobacteria were acquired using Leica LAS X software (Leica Microsystems).

Measurement of Intracellular ROS

To detect intracellular ROS, cells were stained with 10 µM H₂DCFDA (C6827, Life Technologies) for 30 min at 37°C in the dark, and the cells were then incubated in PBS at 37°C for 1 h for recovery. ROS were measured using a microfluorescence reader (Fluoroskan Ascent, Thermo Scientific) at 488/533 (Ex/Em) nm.

Total RNA Extraction

NTPJ-treated Mtb H37Rv was resuspended in 1 ml of Trizol reagent (Life Technologies) and then sonicated at 20 kHz for 5 min (Pulse on: 5 s, Pulse off: 5 s, Amp: 32%) in an ice box [21]. After 10 min of incubation at 37°C, 0.5 ml of chloroform was added to the Trizol reagent mixture and mixed vigorously for 15 s. The cell debris was removed by centrifugation at 10,000 ×g for 10 min at 4°C, and the upper aqueous phase solution was collected in a new tube. Then, 0.5 ml of isopropanol was added to the aqueous phase and incubated at RT for 10 min. RNA was precipitated by centrifugation at 13,000 ×g for 15 min at 4°C. The RNA pellet was washed twice with 70% ethanol, air-dried for 15 min, and then resuspended in 40 µl of DEPC (diethyl pyrocarbonate)-treated water. RNA concentration and purity (A260/A280 nm) were measured using NanoDrop (ND-1000, Qiagen). The total RNA was converted to cDNA using Cyclescript RT Premix (dT20, Bioneer), according to the manufacturer's protocol. The synthesized cDNA was used for real-time PCR (Biomera, UNO Thermoblock).

Quantitative Real-Time PCR (qRT-PCR)

Two-step qRT-PCR was performed with cDNA samples using the Rotor-gene SYBR Green PCR Master Mix (204076, Qiagen). qRT-PCR was performed using the following thermal cycling conditions: initial heat activation at 95°C for 5 min followed by 40 cycles of 95°C for 10 sec, 50°C for 20 sec, and 72°C for 15 sec [22].

Table 1. Oligonucleotide Sequences in this study.

Oligonucleotide name	Sequence of oligonucleotide (5'-3')	
RT 16S rRNA	F:TCC CGC GCC TTG TAC CC	R:CCA CTG GCT TCG GGT GTT A
RT katG	F:CCC ATG GCG CCG GCC CGG CC	R:CGA TGC CGC TGG TGA TCG CG
RT furA	F:AAA CGA TTT TCG GTG CCG TG	R:CGT CCA ACA GGA AGC CGT TA
RT ideR	F:AGT AAC CGT CGA AAC CAC CC	R:ACT TTC TCG ACC TTG ACC GC
RT bfrB	F:ATT TCC TCG TCG GCG AGC AGT TC	R:TCA CGT GCA ACG AAG TTC TC
RT recA	F:ACG TCA AGT GTT CGA GGT CC	R:ACG TCA AGT GTT CGA GGT CC
RT SodA	F:ATG TCG ATT CCG GCA GAT CC	R:CAG TGG AAC CAC CAC CGT TA
RT DoxX	F:GCA CAT CTC GGG TCA GAT CA	R:CTT TTC GTT CAG CAA GAT CG
RT SseA	F:CCC ATA TGC CCG ATT ACC CC	R:GTG TGA GCA CGA ACC AGG TA

F: forward primers, R: reverse primers

Relative gene expression was evaluated using the comparative cycle-threshold method. The mRNA level was normalized to that of 16S rRNA [23]. The primers used in this study are listed in Table 1.

SEM Analysis

For scanning electron microscopy (SEM, SU8230, Hitachi), the treated cells were fixed with 2.5% glutaraldehyde (Sigma-Aldrich, 340855) in 0.1 M phosphate buffer for 24 h. The bacterial pellet was washed three times with phosphate buffer, and then fixed with 1% osmium tetroxide (Sigma-Aldrich, 75632) in 0.1 M phosphate buffer for 1 h and dehydrated in a graded series of ethanol concentrations, and finally with 100% ethanol (3 times). The dehydrated pellets were dried in a critical point dryer (EM CPD300, Leica) at room temperature and coated with gold (5 nm). SEM imaging was performed with Supra 60 VP.

Statistical Analysis

Statistical significance was determined using an unpaired Student's *t*-test. The *p* value was obtained for comparison between the control and each treatment. *p* < 0.05 (*), *p* < 0.01 (**) and *p* < 0.001 (***). ns; not significant.

Results

Physical Characteristics of the Plasma Generated by NTPJ with N₂+H₂O and N₂ Gas

The discharged power and plasma components were analyzed. We found that a stable plasma plume was obtained with power of less than 1.91 W, which was dissipated in the discharge (Fig. 1C). Next, ROS produced in the ambient air by plasma jets with N₂ alone, and N₂+H₂O gas were measured. The emission intensity of OH radicals with the addition of 1.16% in N₂, showed peaks at 306.79 nm and 309.36 nm, while the peak intensities were significantly lower for N₂ gas alone (Fig. 2A). The emission spectrum of the pure nitrogen plasma contained NO radicals and N₂ second-positive system (SPS) only (Fig. 2B). The more dominant OH-related spectra appeared when water vapor was added. We further confirmed the efficiency of our device by determining *E. coli* survival. Because the nutrients present in the growth media act as scavengers for ROS, *E. coli* was resuspended in PBS, treated with plasma jets, and thereafter, immediately plated on LB agar. *E. coli* (10⁵) was completely killed after 1 min of treatment (Fig. 2C). The plasma flame discharged by N₂+H₂O gas killed the bacteria more effectively than that by N₂ gas alone.

Plasma Characteristics in the Mycobacterial Culture Media

We next investigated the physicochemical properties of

Fig. 2. (Color online) Optical emission spectra (OES) of a plasma jet with N₂+H₂O and N₂ gas and colony count assay, shows that plasma inactivates *E. coli* rapidly in a time-dependent manner.

(A) In the ranges of 305–315 nm, (B) 200–600 nm. A higher intensity of OH radicals is detected with the addition of water vapor. (C) Relative survival shows that NTPJ treatment inactivated *E. coli* (5×10^4 cells/0.5 ml) rapidly; the percentage of surviving cells was normalized to that of the untreated cells. The values are mean $\pm$ SD (standard deviation) for three replicates, *p* < 0.05 (*). Unpaired Student's *t*-test was performed between the N₂ and N₂+H₂O treatment. *p* < 0.05 (*).

the plasma-treated 7H9 media, the liquid media used for mycobacterial culture. Changes in temperature, pH, and radical concentrations in the culture, after treatment, were measured. When the distance between the sample and plasma exit was 5 mm, temperature of the area measured by FLIR (BCAM SD infrared thermal imager) after plasma treatment, was between 17.6°C and 36°C, depending on the exposure time (Fig. 3A); the media treated by N₂+H₂O plasma showed slightly lower temperature as compared to the media treated by N₂-only plasma. There were no significant changes in the pH of the media, before or after NTPJ treatment with N₂+H₂O or N₂ gas alone (Fig. 3B). The intensities of OH radicals, hydrogen peroxide concentration, and nitrite concentration in the culture media, which play important roles in bacterial inactivation, were increased

Fig. 3. (Color online) Changes in the temperature, pH and radical concentrations after NTPJ treatment with N₂+H₂O and N₂ gas in 7H9 medium.

(A) Plasma treatment set-up and FLIR thermal image of the 24-well plate surface without exposure (left) and after plasma treatment (right). Colors shown in the images represent the relative temperature of the water surface; the temperature range corresponds to the colors (blue through red) on the color bar shown at the bottom of each image. Temperature of the central region of the well plate is indicated at the upper left corner of each image, (B) pH, and (C) OH radical intensity (fluorescence intensity was normalized to that of the untreated samples), H₂O₂ concentration, and nitrite concentration, after NTPJ treatment with N₂+H₂O and N₂ gas alone, for 1, 3, 5 min, and with 1.5 kU catalase for 5 min, in 7H9 media. Error bars represent the SD. $n = 3$. Unpaired Student's t -test was performed between the N₂ and N₂+H₂O treatment. $p < 0.05$ (*) and $p < 0.01$ (**). ns, not significant.

with a corresponding increase in the NTPJ exposure time (Fig. 3C). Nitric monoxide (NO) radicals from NTPJ may be converted to nitrite (NO₂), which reacts with oxygen in cells and media [24]. Generation of radicals by N₂+H₂O plasma was significantly higher than that by N₂ plasma, except for NO₂. In a parallel experiment, we conducted catalase enzyme scavenging tests in which the production of hydrogen peroxide was significantly decreased by incubation with catalase before plasma treatment (Fig. 3C). Collectively, these results suggest that ROS from N₂+H₂O plasma are generated in significant amounts in 7H9 media.

The Effect of NTPJ Treatment with N₂+H₂O Gas and N₂ Gas Alone, on Mycobacterial Inactivation

Mycobacteria have a thick cell wall comprising diverse lipids and glycolipids, compared to other bacteria [17]. We compared the susceptibility of *E. coli* and rapidly growing nonpathogenic Msm to NTPJ treatment. Both bacteria were resuspended in PBS, followed by treatment with plasma. As shown in Fig. 4A, some of the Msm were found to

survive even after N₂+H₂O plasma treatment for 2 min, in contrast to *E. coli*, suggesting that Msm is more resistant to plasma. Next, the sterilizing effects of plasma, generated with N₂ and N₂+H₂O gas, on Msm were investigated in the 7H9 mycobacterial culture medium. Msm, treated with NTPJ for the indicated time, were plated on 7H10 agar plates immediately (Fig. 4B), and at 24 h (Fig. 4C) after treatment to monitor their survival. As shown in Fig. 4B, approximately 20~40% of the Msm exposed to N₂ gas plasma (depending on the time of exposure) were killed, while 25~75% of the Msm exposed to plasma generated by N₂+H₂O gas, were killed, when immediately plated after exposure. However, it was observed that, when the bacteria were plated 24 h after exposure to N₂+H₂O plasma or N₂ plasma for 5 min, 98% and 80% of the bacteria respectively, were killed (Fig. 4C), indicating that plasma-mediated bacterial killing effects were more apparent after 24 h incubation than immediately after plasma treatment. Also, samples that were preincubated with catalase before N₂ + H₂O plasma exposure for 5 min showed increased

Fig. 4. (Color online) NTPJ with N_2+H_2O and N_2 gas inactivates nonpathogenic *Msm* in a time-dependent manner.

(A) Relative survival of *Msm* versus *E. coli* immediately after NTPJ treatment with N_2+H_2O gas for 0, 1, 2, and 3 min in PBS, (B) 0 h, (C) 24 h after NTPJ treatment with N_2+H_2O and N_2 gas for 0, 1, 3, and 5 min, and with catalase for 5 min in 7H9 media. CFU were plated immediately and at 24 h incubation after treatment on 7H10 solid media supplemented with ADS and were normalized to untreated. $n = 3$. $p < 0.05$ (*) and $p < 0.01$ (**). Unpaired Student's *t*-test was performed between the N_2 and N_2+H_2O treatment. $p < 0.05$ (*) and $p < 0.01$ (**). ns, not significant.

number of bacteria (Figs. 4B and 4C). We concluded that the decrease in the number of surviving bacteria is related to the oxidation of cells and that, the N_2+H_2O plasma jet effectively kills the *Mtb* cells through OH radicals and H_2O_2 . Therefore, the effects of plasma generated with N_2+H_2O gas, on the inactivation of mycobacteria, were investigated in the subsequent experiments. We next investigated the sterilizing effects of plasma on pathogenic *Mtb*. The *Mtb* exposed to N_2+H_2O plasma for 5 min, were plated on 7H10 agar, immediately or after incubation for 1 to 3 days in 7H9 media. As shown in Fig. 5A, 70% of the

virulent *Mtb* H37Rv was killed immediately after treatment, but this proportion increased to 91% after 24 h incubation, when compared to the untreated control. There was no difference in the *Mtb* survival rate among the cells incubated for 1, 2, and 3 days after plasma treatment. The pretreatment of *Mtb* with catalase showed significantly more bacteria than in samples without catalase (Fig. 5A).

Intracellular ROS concentration is an important factor that exerts antimicrobial effects. Therefore, we estimated the intracellular ROS level of *Mtb*, using the H_2DCFA fluorescence dye assay (Fig. 5B). After plasma treatment for

Fig. 5. (Color online) NTPJ with N_2+H_2O gas inactivates pathogenic *Mtb* H37Rv cells.

(A) Relative survival was normalized to untreated cells at 0, 1, 2, and 3 days incubation, (B) ROS levels inside *Mtb* cells were measured using H_2DCFDA fluorescent dye in 7H9 growth media after NTPJ treatment for 5 min and with catalase for 5 min. Fluorescence was measured consecutively and was normalized to that of untreated cells. Error bars represent the SD. $n = 3$. $p < 0.05$ (*) and $p < 0.01$ (**). Unpaired Student's *t*-test was performed between N_2+H_2O plasma exposure for 5 min with pre-incubated catalase and N_2+H_2O treatment. $p < 0.05$ (*) and $p < 0.01$ (**). ns, not significant.

5 min, the ROS level within the Mtb cells was found to increase with a corresponding increase in incubation time. However, the increase in the ROS level within the Mtb cells was less apparent when catalase was added to the sample. At 2 h of incubation, the ROS level in the plasma-treated Mtb was increased by 2.3-folds, compared to that in the untreated Mtb, and was significantly higher than that in the catalase-added samples. Therefore, these data suggest that OH radicals, generated by NTPJ, convert to hydrogen peroxide, which enhances intracellular ROS production, playing a role in killing of the bacteria.

Plasma Treatment Disrupts Mycobacterial Wall Integrity

We observed through SEM that radicals from a N_2+H_2O plasma clearly damaged cell-wall structure of the mycobacteria (Fig. 6). The cell-wall integrity was also found to be protected by the addition of catalase. There was no remarkable difference in plasma-mediated damage inflicted on non-pathogenic Msm and pathogenic Mtb. To further confirm the effect of plasma on bacteria, the N_2+H_2O plasma-exposed Msm or Mtb were stained with the live/dead bacteria staining kit. Dead cells allow the entry of the impermeable PI dye, such that it binds DNA and emits red fluorescence. The untreated Msm and Mtb were stained green (Figs. S1 and 7). Msm were stained green immediately, after 3 min-treatment, however, those bacteria that were subjected to 5 min-treatment, stained red (Fig. S1). The bacteria that stained red and green simultaneously, showed yellow color in a merged plate, indicating that they were dying bacterial cells. Mtb treated with plasma for 5 min were stained red and green, but the number of dead bacteria stained with PI was significantly increased by plasma treatment compared to the untreated bacteria (Fig. 7). The number of red stained-bacteria decreases by pre-incubation with catalase before plasma exposure, in comparison to plasma treated-bacteria not incubated with catalase. Therefore, these data indicate that radicals generated by NTPJ may play a critical role in damaging the membranes and cell walls of mycobacterial cells.

Expression of Genes Related to ROS Stress Responses in N_2+H_2O Plasma-Treated Mtb

To further understand the intra-bacterial responses to NTPJ, the expression of genes involved in the stress response against ROS in Mtb was analyzed by qRT-PCR. Because intracellular ROS were much higher after 2 h incubation than immediately after plasma exposure (Fig. 5B), total RNA from N_2+H_2O plasma-exposed Mtb was extracted after 2 h of incubation. Fig. 8A indicates that

Fig. 6. (Color online) Effect of NTPJ with N_2+H_2O gas on mycobacterial wall integrity was evaluated by SEM.

Untreated cells, immediately after 5 min exposure, and cells exposed to a 5 min treatment under N_2+H_2O gas in the presence of catalase. NTPJ treatment enhances the disruption of the cell wall. The black arrows indicate the cell rupture of mycobacteria.

transcriptional response to plasma treatment is associated with detoxification of radicals through the membrane-associated oxidoreductase (SseA, DoxX, SseA) component of the cell membranes [25], which was enhanced compared to that in the untreated Mtb. SseA (thiol oxidoreductase, ~ 9-fold) and DoxX (integral membrane protein, ~ 6-fold) showed higher expression. In contrast, the expression of superoxide-detoxifying enzyme (SodA, ~1.8), which is a major antioxidant in Mtb, was much lower compared to that of DoxX and SseA. As expected, addition of catalase to the media before plasma exposure significantly decreased the plasma-mediated expression of these genes. As a positive control, Mtb treated with H_2O_2 showed a gene expression pattern similar to that obtained with plasma exposure (Fig. 8A). Moreover, the expression of SseA (~21-fold) and DoxX (~ 8-fold) genes was enhanced with H_2O_2 (0.1 mM) treatment, compared to that in the untreated Mtb cells. Additionally, we investigated the expression of several

Fig. 7. (Color online) NTPJ with N_2+H_2O gas attacks the cell membrane in Mtb H37Rv cells and leads to gradual cell leakage. BacLight Live/Dead viability assay essentially tests the intact and damaged membranes in Mtb cells, and thus dying bacterial cells show yellow fluorescence, and dead cells show red fluorescence. The scale bar is 1 μ M. Untreated cells, immediately after 5 min exposure, and exposure for 5 min with pre-incubated catalase. The column on the right shows the merged image of SYTO (green dye, Ex 480/500 nm) and PI (red, 490/635 nm). The scale bar is 1 μ M.

other genes related to iron regulation (*furA*, *ideR*), iron storage (*bfrB*), transcriptional regulation (*katG*), and DNA repair (*recA*) in plasma exposed- or H_2O_2 -treated-Mtb. N_2+H_2O plasma or H_2O_2 induced the expression of these tested genes in Mtb (Fig. 8B). Catalase also suppressed the

plasma-mediated upregulation of these genes. Collectively, these results suggest that NTPJ enhances the expression of genes related to thiol oxidoreductase, iron-homeostasis, and stress responses in Mtb cells, indicating a need for detoxification.

Fig. 8. (Color online) Identifying the defense systems against ROS in N_2+H_2O plasma jet.

Total mRNA was isolated using Trizol reagent, qRT-PCR analysis of Mtb cells at 2 h incubation after 5 min treatment, with catalase for 5 min, and 0.1 mM H_2O_2 (2 h) was performed. (A) Expression of membrane-associated reductase genes (*DoxX*, *SodA*, *SseA*) (B) Expression of iron homeostasis genes *furA*, *ideR*, and *bfrB*, transcriptional regulator gene *katG*, and DNA repair gene *recA*. Data are representative of at least two different experiments performed in triplicate. Error bars show standard deviations from the mean. $n = 3$.

Discussion

Among the ROS, the hydroxyl OH is toxic to cells and causes cell death [26, 27], as bacteria do not contain scavenging enzymes for OH radicals [28]. More recently, it has been reported that OH radicals from a NTPJ also increase cell apoptosis, and showed selectivity between cancer cells and normal cells [29]. In this study, we found that the plasma generated by a NTPJ device effectively inactivated mycobacteria. Another report showed that *E. coli* was completely inactivated for 60 sec exposure to floating-electrode dielectric-barrier discharge (FE-DBD) [30]. The Mtb cell envelop differs substantially from the cell wall structure of common bacteria and is a highly lipophilic barrier that contributes to resistance against common antibiotics [31]. It is well known that the lipid content of Mtb constitutes about 60% of the dry weight of the bacteria. Therefore, mycobacteria are more resistant to plasma than *E. coli*, as shown in Fig. 4A. We have clearly demonstrated that the plasma jet device generates a stable plasma jet with micro-discharges in a porous layer [10]. As shown in Fig. 1C, we were able to obtain a stable plasma jet at 1.91 W, through the generation of steady discharge voltage and current pulses during plasma discharge. The process temperature of the plasma jet was 17–36°C during the time-dependent treatment, indicating that heat is not the only factor responsible for effective antibacterial action. Further, we demonstrated that OH radical production is higher when a mixture of H₂O and N₂ is used in the plasma jet compared to N₂ alone [19]. In this study, the plasma jet containing a mixture of 1.16% H₂O and N₂ produced more dominant OH radicals compared to that with N₂ alone, as indicated by the more effective bactericidal activity demonstrated against *E. coli* and mycobacteria.

To further understand the antimicrobial activity of ROS (OH and H₂O₂) in mycobacteria, we investigated if there was a change in intracellular ROS concentration after plasma treatment. We observed that after 2 h incubation, intracellular ROS were increased by 2.3 fold (Fig. 5B). It is reported that intracellular concentrations of hydrogen peroxide (less than 1 μM) are toxic for *E. coli* [32]. We envisage that the hydrogen peroxide produced (48 μM concentrations, Fig. 3C) in growing media, diffuses in the water, penetrating and interacting with Mtb cells inside the media. The hydrogen peroxide exhibits strong antibacterial properties by inducing thiol oxidation, which subsequently damages enzymes and proteins [33, 34]. We therefore conclude that stress due to hydrogen peroxide can enhance the rate of bacterial inactivation. Our finding that the

sterilizing effect of N₂ + H₂O plasma on Mtb cells in growth media was more substantial after 24 h rather than immediately (Fig. 4B), further suggests that ROS production in plasma persists over the course of time to trigger bactericidal activity inside the mycobacterial cells (Figs. 4C and 5A).

ROS generated in plasma directly attack microbial DNA, lipids, and proteins in cell membranes, leading to cell damage [35]. Plasma treatment enhances expression of genes related to ROS stress in bacteria [36], however, there is no report on the effect of plasma-induced radicals on the expression of SseA and DoxX during detoxification of bacteria. In this study, significant up-regulation of SseA and DoxX were observed in mycobacteria (Fig. 8A). The major role of SseA and DoxX is to promote resistance to ROS stress that disrupts cytosolic thiol homeostasis [25], whereas SodA does not directly influence thiol recycling; however, this protein is a key player in thiol homeostasis as a component of the membrane-associated oxidoreductase complex (MRC). Our data also showed that SodA was not strongly enhanced, suggesting that ROS may affect thiol homeostasis. Optical spectroscopy data showed that the plasma jet did not generate superoxide radicals (O₂^{•−}), therefore, we can speculate that the hydroxyl radicals from the plasma jet cause the formation of hydrogen peroxide, which then oxidizes iron-sulfur (Fe-S) proteins, resulting in increased ferrous ion concentration. Notably, the ferric uptake regulator, *furA*, was more enhanced compared to other genes (*ideR*, *bfrB*, *katG*) after NTPJ treatment (Fig. 8B). It may be envisaged that an increased production of ferrous ions results in the generation of reactive oxygen species, which in turn, triggers the detoxification of cells. In line with our finding, many studies have reported the involvement of iron-regulation genes (*furA*, *ideR*) in detoxification of exogenous or endogenous oxidants [37, 38]. In this regard, OH radicals play a critical role in antibacterial activity of mycobacteria. In the current study, 0.1 mM H₂O₂ treatment also confirmed a similar pattern of up-regulation of genes such as DoxX, SseA, and *furA* which are important players in promoting resistance to ROS stress.

In our study, membrane leakage was observed (Figs. 6 and 7) when water vapor was used in the plasma discharge, suggesting that ROS in plasma interact with the membranes of the Mtb cell wall which are their primary cell barrier, inducing loss of membrane integrity. Taken together, these data suggest that the OH radicals, generated by NTPJ, convert to ROS within the bacteria and play a bactericidal role.

The NTPJ device used in this study was specifically

designed for production of large amounts of hydroxyl radicals and hydrogen peroxide. The OES results (Figs. 2A–2B) suggest that NO radicals, OH radicals, and the N_2 second-positive system (SPS) were significantly produced, but the generation of OH radicals is much more compared to NO radicals. The excited nitrogen molecules in a metastable level of $N_2(A_3\Sigma_u^+)$ dissociate water molecules, and generate hydroxyl radicals and hydrogen atoms, which is one of the most important reactive species [6, 19]. Plasma discharge in the nitrogen gas mixed with water molecules produces NH radicals, but the density of NH radicals is 5 orders in magnitude less than the hydroxyl density [39], indicating that hydroxyl in the nitrogen plasma dominates over other species except hydrogen peroxides. The hydrogen peroxide density is one order of magnitude higher than the hydroxyl density [39]. The results of this study are meaningful for pulmonary-disease control in the biomedical field. The NTPJ treatment sterilizes effectively NTM species in water such as *M. avium*, *M. intracellulare*, and *M. abscessus*, which are responsible for pulmonary disease infections.

In summary, this is the first study to report the application of NTPJ in mycobacterial inactivation. Using 1 slm of nitrogen-water vapor (1.16%) gas flow and 1.91 W of discharge power at atmospheric pressure, we obtained a stable plasma jet for efficient sterilization. The plasma jet with N_2+H_2O gas killed the bacteria more effectively than that with N_2 gas only. The OH radicals generated by NTPJ convert to hydrogen peroxide, which enhances intracellular ROS production, that play a major role in killing bacteria. Radicals from NTPJ disrupt mycobacterial cell wall integrity, leading to enhanced expression of genes related to thiol oxidoreductase, iron-homeostasis, and stress responses in Mtb cells, owing to a need for detoxification against ROS stress following exposure to the OH radicals.

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Conflict of Interest

The authors have no financial conflicts of interest to declare.

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