Control of multidrug-resistant planktonic Acinetobacter baumannii: biocidal efficacy study by atmospheric-pressure air plasma

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Abstract
In this research, an atmospheric-pressure air plasma is used to inactivate the multidrug-resistant Acinetobacter baumannii in liquid. The efficacy of the air plasma on bacterial deactivation and the cytobiological variations after the plasma treatment are investigated. According to colony forming units, nearly all the bacteria (6-log) are inactivated after 10 min of air plasma treatment. However, 7% of the bacteria enter a viable but non-culturable state detected by the resazurin based assay during the same period of plasma exposure. Meanwhile, 86% of the bacteria lose their membrane integrity in the light of SYTO 9/PI staining assay. The morphological changes in the cells are examined by scanning electron microscopy and bacteria with morphological changes are rare after plasma exposure in the liquid. The concentrations of the long-living RS, such as H2O2, NO3− and O3, in liquid induced by plasma treatment are measured, and they increase with plasma treatment time. The changes of the intracellular ROS may be related to cell death, which may be attributed to oxidative stress and other damage effects induced by RS plasma generated in liquid. The rapid and effective bacteria inactivation may stem from the RS in the liquid generated by plasma and air plasmas may become a valuable therapy in the treatment of infected wounds.

Keywords: atmospheric-pressure air plasma, multidrug-resistant bacteria, Acinetobacter baumannii, inactivation, reactive species

(Some figures may appear in colour only in the online journal)

1. Introduction

Acinetobacter baumannii is a gram-negative bacteria species which has emerged as an important opportunistic nosocomial pathogen in hospitals [1]. The prevalence of A. baumannii is
due to its biological characteristics, such as stability, which enables the bacteria to survive in extreme situations of temperature, moisture, and acidity and in the existence of common detergents [2]. Moreover, owing to the inappropriate use and abuse of antibiotics, the antimicrobial resistance of A. baumannii species has increased significantly in recent decades. In addition to the external environment, its capacity to respond rapidly to selective environmental pressure also contributes to the antimicrobial resistance of A. baumannii, which includes enzymatic degradation of β-lactams, enzymatic modification of aminoglycosides, interference with target site binding of quinolones, and a variety of changes in outer membrane proteins [3]. The multi-drug resistant A. baumannii, which causes a wide range of infections of the skin, soft tissues, and respiratory systems, has emerged as an important opportunistic pathogen [4] infecting people with compromised immune response such as patients in the intensive care unit [5]. Apart from increased hospitalization and the associated costs, there are serious consequences such as increased morbidity and mortality [6]. The prevalence of multidrug-resistant A. baumannii has increased to worrisome levels because gold-standard carbapenem antibiotics and nearly all types of antibiotics are not sensitive to multidrug-resistant A. baumannii [7]. Consequently, new and effective strategies are required to combat the multidrug-resistant bacteria.

Cold atmospheric-pressure plasmas have been used in bacteria inactivation [8, 9], cancer therapy [10, 11], blood coagulation [12], and cell proliferation [13]. Owing to the flexible room temperature operation, high effective eradication of microorganisms, absence of residual toxic substances, repeatable nature and excellent reproducibility [14], non-thermal atmospheric-pressure plasmas have attracted increasing attention in the treatment of wounds [15], sterilization of medical instruments [9], as well as food sanitation [16]. Although cold atmospheric-pressure plasmas have shown an efficient killing effect against bacteria, it seems to induce little or no damage to mammalian cells due to the difference in structure and morphology between prokaryotes and mammalian cells, which means that it is possible to use plasma in various medical applications [17]. In the generated plasmas, reactive charged and neutral active species, ultraviolet light, electrons, and other reactive species (RS) including OH, O, O3, nitric oxide, superoxide, hydrogen peroxide, and singlet oxygen are present [18–20]. As non-thermal plasmas can generate these active species to sterilize surfaces, the technique offers antimicrobial capability [21]. In clinical use, infected wounds are usually moist and covered with interstitial fluids and bacteria and so the ability to inactivate bacteria in solutions is imperative. Although energetic particles created by plasmas cannot interact directly with bacteria in the liquid [22], they can react with water to initiate a sequence of physical-chemical reactions [23]. The reaction products can be classified into two categories: short-living RS, including OH−, O2, and singlet oxygen, and long-living RS, including H2O2 and O3. Both play important roles in bacteria inactivation in addition to heat, UV and electrical fields in the plasmas [24–26].

In spite of recent advances in plasma technologies, the interactions involving plasma-triggered RS in aqueous solutions and the concomitant structural and functional changes of the microbes on the cellular level are not yet well understood. Furthermore, research into plasma deactivation of multidrug-resistant A. baumannii in liquids has been limited. In this study, the effectiveness of plasma inactivation of multidrug-resistant A. baumannii in the planktonic form and subsequent cytochemical variation after the plasma exposure including cell membrane integrity, cell metabolic capacity, intracellular reactive oxygen species (ROS) level, and cell morphological changes are investigated. Moreover, the reaction products produced by the plasma in water and their relationship with bacteria inactivation are evaluated.

2. Materials and Methods

2.1. Atmospheric-pressure air plasma

The atmospheric-pressure air plasma for bacterial inactivation was constructed in our laboratory and the details are shown in figure 1. The length of the wire after the resistor is about 5 cm. The length and diameter of the needles are 2 cm and 1 mm respectively. Similar types of configuration are also reported by other articles [27–31]. In this study, the six fine needles were driven by a high voltage DC and the bacterial suspension served as the ground electrode. The plasma was generated between high voltage and bacterial suspension without any supply of an external gas. The resistors R in figure 1 limited the discharge current. The input voltage was 10 kV. The discharge current was 5 mA (peak value). The gas temperature of the air plasma was about 300 K due to the low input power.

2.2. Cultivation of A. baumannii suspensions and plasma treatment

The multidrug-resistant A. baumannii was provided by the First Affiliated Hospital of Anhui Medical University, and the antibiotic resistance details are showed in table 1. The bacteria were cultured in LB (Luria-Bertani) medium overnight at
plates were counted to determine the plasma inactivation under atmospheric conditions and the overnight culture diluted samples were spread on the LB agar plates with sterile overnight, the culture was diluted to 10⁶ CFU ml⁻¹ followed by exposure, the bacterial suspensions were stained by the SYTO9 stain membrane-intact bacteria exhibited green fluorescence, whereas membrane-damaged bacteria showed red fluorescence. After plasma treatment, inactivation of A. baumannii was determined by the plate counting method. The bacteria suspensions were diluted to the appropriate concentrations by serial 10-fold attenuations with sterile water. 100 μl of the diluted samples were spread onto the LB agar plates with sterile triangular glass rods. The plates were incubated at 310 K for 12 h under atmospheric conditions and the overnight culture plates were counted to determine the plasma inactivation efficiency. Each experiment was performed three times.

2.3. Inactivation of A. baumannii

After the plasma treatment, inactivation of A. baumannii was evaluated by the plate counting method. The bacteria suspensions were diluted to the appropriate concentrations by serial 10-fold attenuations with sterile water. 100 μl of the diluted samples were spread on the LB agar plates with sterile units per milliliter. 2 ml of the bacteria suspension were added into a petri dish (35 mm diameter). Then the dishes were exposed to the non-thermal atmospheric-pressure plasma for 30 s, 1, 3, 5, and 10 min, respectively.

2.4. Fluorescent staining

2.4.1. Membrane integrity. The membrane integrity was determined by the LIVE/DEAD BacLight™ bacterial viability kit (L7012, Invitrogen, USA) following the prescription provided by the manufacturer [32]. The SYTO9 stain membrane-intact bacteria exhibited green fluorescence, whereas membrane-damaged bacteria showed red fluorescence with propidium iodide. After the plasma exposure, the bacterial suspensions were stained by the bacterial viability kit and incubated for 15–20 min at room temperature. Afterwards, the samples were analyzed with a microplate reader and fluorescence microscope. The excitation/emission wavelengths for the SYTO 9 stain and propidium iodide were 485/530 nm and 485/630 nm respectively.

2.4.2. Cellular metabolic capacity of A. baumannii. Resazurin (blue and nonfluorescent) was reduced to resorufin (purple and highly fluorescent) by intracellular enzymes and resazurin was used to assess the level of cellular metabolism [33]. In the in vitro toxicology assay, resazurin (Sigma) was employed to detect the bacterial metabolic ability following the prescription provided by the manufacturer. After the plasma treatment, resazurin was added and cultured with the bacteria. The samples were analyzed by a microplate reader (Varioskan Flash) at excitation/emission wavelengths at 560/590 nm.

2.4.3. Intracellular ROS concentration. The intracellular ROS concentration was assessed by the ROS Assay Kit (Beyotime). The primary constituent of the ROS assay kit was DCFH-DA (2,7-dichlorodihydro fluorescein diacetate) which could pass through the cell membrane and be transformed into non-fluorescent DCF (dichlorofluorescin) through enzymatic hydrolysis reaction. The DCFH could not pass through the cell membrane and the probe could be easily loaded into the cells. The intracellular ROS oxidized the non-fluorescent DCFH producing fluorescent DCF [34]. The intracellular ROS was evaluated based on the fluorescence level of DCF. After the plasma exposure, the bacteria were mixed with the diluted DCFH-DA solution and incubated in darkness for 20 min at 37 °C. After incubation, the samples were acquired by centrifugation and washed with deionized water to remove excess DCFH-DA. The samples were analyzed on a microplate reader (Varioskan Flash) at excitation/emission wavelengths at 488/525 nm.

2.5. RS in plasma-treated liquid

Using the spectrophotometric method, the concentration of long-living RS including H₂O₂, O₃, and NO₃ in the liquid after plasma treatment was evaluated using the PhotoLab 6100 (WTW) with test kits 18 789, 00 607, and 09 713 [35, 36].

2.6. Scanning electron microscopy

The morphological changes in the plasma-treated bacteria were examined by scanning electron microscopy (SEM). The SEM images were acquired on the S4800 SEM (Hitachi, Japan) at 15,000X after gold-palladium coating.

2.7. Statistical analysis

Every experiment was carried out in triplicate. The results were showed as mean value ± standard deviation (SD). Statistical analyses were performed using the ANOVA test, using the SPSS Statistics 21.0 software. The significance level was set as less than 0.05.

3. Results and discussion

3.1. Time-dependent response of planktonic cultures to the plasma treatment

To demonstrate the efficacy of the air plasma on A. baumannii, the colony count assay is adopted. Figure 2 shows the logarithmic survival rate of the bacteria following exposure to

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Sensitivity</th>
<th>Results (mg l⁻¹)</th>
<th>Methods</th>
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<tbody>
<tr>
<td>Cefepime</td>
<td>R</td>
<td>≥ 64</td>
<td>MIC</td>
</tr>
<tr>
<td>Tobramycin</td>
<td>R</td>
<td>≥ 16</td>
<td>MIC</td>
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<tr>
<td>Levofloxacin</td>
<td>R</td>
<td>≥ 8</td>
<td>MIC</td>
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<tr>
<td>Gentamicin</td>
<td>R</td>
<td>≥ 16</td>
<td>MIC</td>
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<tr>
<td>Ampicillin</td>
<td>R</td>
<td>≥ 32</td>
<td>MIC</td>
</tr>
<tr>
<td>Ceftriaxone sodium</td>
<td>R</td>
<td>≥ 64</td>
<td>MIC</td>
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<tr>
<td>Cefotetan</td>
<td>R</td>
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<td>Cefazolin</td>
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<td>Imipenem</td>
<td>R</td>
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<tr>
<td>Aztreonam</td>
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<tr>
<td>Ciprofloxacin</td>
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<td>Cefazidime</td>
<td>R</td>
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37 °C with a rotational speed of 150 rpm, and after culturing overnight, the culture was diluted to 10⁶ CFU ml⁻¹ (colony forming units per milliliter). 2 ml of the bacteria suspension were added into a petri dish (35 mm diameter). Then the dishes were exposed to the non-thermal atmospheric-pressure plasma for 30 s, 1, 3, 5, and 10 min, respectively.
the air plasma. The original *A. baumannii* population is approximately 6.5 log_{10} CFU ml^{-1} and with increasing treatment time, the survival rate of *A. baumannii* decreases gradually. After 3 min, the surviving was about 6 log_{10} CFU ml^{-1} and nearly 100% (6-log) reduction was achieved after 10 min.

The surviving curve of *A. baumannii* exhibits time dependence. As time increases, the decimal logarithm of surviving bacteria decreases gradually. The air plasma can generate UV light emission, energetic particles, OH and O radicals in the gas–liquid interface. While these species can be blocked by the liquid, some are nevertheless captured immediately by water molecules to form RS by chemical reactions, so the RS may have inactivation ability. The concentration of RS generated in the liquid increases with plasma treatment time, and there is enough time for the RS to spread and inactivate the bacteria, which results in a gradual decrease of bacteria population.

### 3.2. Metabolic activity of bacteria after the plasma treatment

Resazurin (blue and nonfluorescent) can be reduced to resorufin (purple and highly fluorescent) by intracellular enzymes and so resazurin is used to assess cellular metabolism. The resazurin assay is designed to discern dead and viable cells and only viable cells convert resazurin into its fluorescent product as a metabolic marker, which can be quantified as stated in the Materials and Methods section [30]. The metabolic activity of the bacteria before and after plasma exposure is monitored by resazurin. As shown in figure 3, the percentage of bacteria with metabolic capacity is time-dependent and decreases with plasma exposure time. The fraction of surviving cells determined by the resazurin assay is greater than that by the colony count method (figure 2). After exposure for 180 s and 300 s, the resazurin survival fractions are 73.64% and 28.34%, respectively, whereas the standard plate count survival fractions at the same time point are 47.09% and 0.11%.

The colony count method is the most widely used technique in bacteriology to demonstrate bacterial culturability by counting the bacterial colonies grown on medium from cell division. However, the resazurin assay is utilized to assess cellular metabolism, which could differentiate ‘live’ cells with metabolic capacity including those in the dormancy state but without culturability to form colonies. After plasma treatment of 300 s, the colony count result (figure 2) indicated that 99.89% of the cells may die or lose the ability to reproduce and thereby form colonies, whereas the results determined by resazurin assay (figure 3) indicate that 28.23% of the total bacterial population are still alive with basic metabolic capacity. The difference may indicate that the plasma-exposed bacteria may be converted to the viable-but-non-culturable (VBNC) state [37]. The VBNC state occurs when bacteria face environmental stress such as oxidative stress, desiccation, starvation, osmotic stress, and other adverse conditions [38]. It has been reported that non-thermal plasmas also convert micro-organisms into the VBNC state [8, 39, 40], acting as an environmental stress. Since the RS in the liquid inhibit bacterial growth, RS such as OH⁻, H₂O₂, NO₃⁻, and O₃ can be classified as environmental stress. VBNC cells have intact membranes that contain intact genetic information. In addition, VBNC cells are active in metabolism, capable of respiration and expressing genes [41, 42]. Plasma treatment might have inhibited the expression profile of genes related to bacterial culturability or metabolic capacity to different extents, which could be one of the main reasons for the differences in bacterial percentages post-treatment [43].

### 3.3. Bacterial membrane integrity of *A. baumannii*

The LIVE/DEAD assay (Molecular Probes) is utilized to distinguish cells with ruptured membranes based on propidium iodide (PI) uptake. SYTO 9 is a nucleic acid dye which can label all the bacteria, while propidium iodide (PI) only penetrates cells with injured membranes. When both dyes are present, PI can displace the SYTO 9 in the cells so that damaged membranes are stained red. The percentage of intact
membrane bacteria is shown in figure 4 and the microscopic images of the bacteria before and after plasma exposure are depicted in figure 5. With increasing plasma exposure time, the percentage of bacteria with intact membranes decreases.

The inactivation results obtained from conventional cultivation are normally lower than those obtained by the LIVE/DEAD staining method. For example, after exposure for 10 min, nearly 100% reduction is observed from the standard plate counting method (figure 2) but the percentage of bacteria losing intact membranes is 86% according to the LIVE/DEAD staining assay (shown in figure 5). Loss of membranes translates into bacterial death and the different plate counting and LIVE/DEAD staining assay results indicate that some bacteria with intact membranes may enter the non-cultivated state. However, the percentage with metabolic capacity (figure 3) and the percentage with intact membrane are similar after the same plasma exposure time, suggesting that bacteria with intact membranes may have metabolic capacity.

3.4. Surface morphology of bacteria after the plasma treatment

Figure 6 shows the SEM micrographs of A. baumannii before and after the plasma treatment. With increasing exposure time, the amount of bacteria with morphological changes go up slightly. After 10 min, nearly all the bacteria are dead or enter the non-culturable state (figure 2). 86% of the bacteria lose membrane integrity (figure 5) although bacteria with morphological changes are rare. This phenomenon indicates that morphological changes are not the only mechanism in which cells are affected by the plasma. In fact, there is no clear correlation between cell death and morphological changes after plasma exposure. Morphological changes may lead to cell death, but cell death does not mean that the cells have morphological changes. It has been shown that bacteria undergo morphological changes after plasma treatment under dry conditions due to etching by oxygen radicals in the plasma [44, 45]. However, in this study, the bacteria are kept away from etching by oxygen radicals because they are in the liquid [32]. Hence, the morphological changes of the bacteria in the liquid treated by the plasma are different from those under dry conditions.

3.5. Intracellular ROS of the bacteria

The intracellular ROS are monitored to elucidate the relationship between the bacteria deactivation process and intracellular ROS concentrations. Figure 7 displays the variations in the intracellular ROS after air plasma exposure. The intensity of the intracellular ROS exhibits an up-and-down trend. Bacteria have their own mechanisms to confront oxidative stress in vivo. The bacterial catalase, catalase-peroxidase, and superoxide dismutase play a crucial role in protecting the bacteria from ROS damage in vivo [46, 47]. The air plasma producing exogenous ROS can damage the external cellular structure and penetrate the cells. However, bacteria may not have enough time to produce sufficient enzymes to protect themselves from oxidative stress and so the balance of intracellular ROS is disrupted and cells are not effectively repaired. On the heels of more cell membrane damage, the DCF probes cannot be totally encased but instead leak out. The intracellular ROS decrease after plasma exposure for 3 min.

3.6. RS generated in liquid after plasma exposure

The air plasma produces electrons, ions, and radicals and, on account of the protection by the liquid, the active species produced by air plasma may not inactivate bacteria directly. The electrons, ions, and radicals can, however, react with water to initiate a sequence of chemical reactions to produce RS including long-living ones such as H2O2, NO3, and O3 as well as short-living ones including OH−, O2−, NO, and ONOO−. H2O2 is generated by the following reactions [48]:

\[ \text{H}_2\text{O} \rightarrow \text{H} + \text{OH} \]  
\[ \text{OH} + \text{OH} \rightarrow \text{H}_2\text{O}_2. \]  

The following reactions produce ozone which dissolves in the liquid [49, 50]:

\[ \text{O} + \text{O} + \text{M} \rightarrow \text{O}_2 + \text{M} \quad \text{[M = third body]} \]  
\[ \text{O} + \text{O}_2 + \text{M} \rightarrow \text{O}_3 + \text{M}. \]  

Generation of nitrate ions (NO3) proceeds by the following reactions [50, 51]:

\[ \text{N}_2 + \text{e}^- \rightarrow 2\text{N} + \text{e}^- \]  
\[ \text{N}_2 + \text{O} \rightarrow \text{NO} + \text{N} \]  
\[ \text{N} + \text{O} \rightarrow \text{NO} \]  
\[ 2\text{NO} + \text{H}_2\text{O} + \text{O}_3 \rightarrow 2\text{HNO}_3 \]  
\[ \text{NO} + \text{O}_3 \rightarrow \text{NO}_2 + \text{O}_2 \]  
\[ \text{NO}_2 + \text{OH} \rightarrow \text{HNO}_3. \]
On account of the transitory half period and high reactivity, it is difficult to detect the concentration of short-living RS in liquid induced by plasma. Therefore, only long-living RS, comprising $\text{H}_2\text{O}_2$, $\text{O}_3$, and $\text{NO}_3^-$ in solution, are measured after the air plasma treatment in this study. As shown in figure 8, the $\text{H}_2\text{O}_2$, $\text{O}_3$, and $\text{NO}_3^-$ concentrations increase with exposure time and after 10 min, the concentration of $\text{NO}_3^-$ soared to $307.6 \text{ mg l}^{-1}$. In contrast, the concentrations of $\text{H}_2\text{O}_2$ and $\text{O}_3$ increased to $49.2 \text{ mg l}^{-1}$ and $1.6 \text{ mg l}^{-1}$, respectively.

Some of the plasma-produced RS can be created in cells to serve as cell signaling molecules and perform biological functions at low concentrations [52, 53]. At high concentrations, RS can react with the cell membrane and intracellular structure due to its high reactivity. For example, $\text{H}_2\text{O}_2$ takes part in intracellular signal transduction at low concentrations,

![Figure 5. SYTO 9/PI stained images showing the integrity of bacterial membrane structure.](image-url)
while high concentrations of H$_2$O$_2$ are related to DNA, lipid and protein damage. OH reacts non-selectively with cell membranes and many intracellular structures including DNA, lipids, proteins, amino acids, and sugars [54]. Short-living RS in liquid induced by the plasma may have a microsecond or nanosecond half-life but they can also be generated from reactions between long-living RS. In some cases, H$_2$O$_2$ decomposes to form OH [55] and ONOO$^-$ is generated by the reaction between hydrogen peroxide (H$_2$O$_2$) and nitrite O$_2$ in an acidic solution [56]. ONOO$^-$ diffuses across phospholipid membranes freely and reacts directly with amino acids such as cysteine, tryptophan, and methionine [54]. These reactions finally lead to the loss of cell membrane integrity and cell

Figure 6. SEM micrographs of A. baumannii after the plasma treatment.

Figure 7. Intracellular ROS of the bacteria after the plasma treatment.

Figure 8. Concentrations of RS in the liquid.
functions [55] as described in sections 3.2 and 3.3. Further studies are required to determine the relationship between bacterial inactivation and short-living RS in detail.

4. Conclusion

The air plasma shows significant antimicrobial effects on multidrug-resistant A. baumannii and nearly a 100% (6-log) reduction is observed by the colony counting assay after plasma exposure for 10 min. However, the resazurin assay shows that 7% of the bacteria retain metabolic capacity after the same plasma exposure and the difference may indicate that some bacteria may enter a physiologically VBNC state to protect themselves from environmental stress. The RS in the liquid generated by the plasma may contribute to the stress. The decline in cellular membrane damage is similar to the decline in cellular metabolic capacity, indicating that some bacteria with intact membranes may be converted to the VBNC state by the plasma. SEM reveals morphological changes after plasma exposure but they are not the only cause of cell death. The concentrations of long-lived RS plasma produced in the liquid such as H2O2, O3, and NO3 increase and they destroy the cell membrane and hamper metabolism of the bacteria by penetrating the cells to disrupt the intra-cellular ROS balance finally resulting in cell death.

Acknowledgments

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