Analysis of the antimicrobial effects of nonthermal plasma on fungal spores in ionic solutions

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The antimicrobial efficiency of reactive species-based control strategies is significantly affected by the dynamics of reactive species in the biological environment. Atmospheric-pressure nonthermal plasma is an ionized gas in which various reactive species are produced. The various levels of antimicrobial activity may result from the dynamic interaction of the plasma-generated reactive species with the environment. However, the nature of the interaction between plasma and environments is poorly understood. In this study, we analyzed the influence of the ionic strength of surrounding solutions (environment) on the antimicrobial activity of plasma in relation to the plasma-generated reactive species using a model filamentous fungus, Neurospora crassa. Our data revealed that the presence of sodium chloride (NaCl) in the background solution attenuated the deleterious effects of plasma on germination, internal structure, and genomic DNA of fungal spores. The protective effects of NaCl were not explained exclusively by pH, osmotic stability, or the level of reactive species in the solution. These were strongly associated with the ionic strength of the background solution. The presence of ions reduced plasma toxicity, which might be due to a reduced access of reactive species to fungal spores, and fungal spores were inactivated by plasma in a background fluid of nonionic osmolytes despite the low level of reactive species. Our results suggest that the surrounding environment may affect the behavior of reactive species, which leads to different biological consequences regardless of their quantity. Moreover, the microbicidal effect of plasma can be synergistically regulated through control of the microenvironment.

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surfaces than on skin [24]. When applying plasma to microbes contaminating food, various levels of sterilization efficiency have been observed depending on food properties, moisture content, and surface texture [25,26].

Plasma’s functional diversity may be derived from the action of reactive species generated by plasma [27]. The dynamics of reactive species in various environments is critical for understanding the mode of plasma action. Reactive species can alter environmental factors, or the environment can modulate the level of reactive species [28]. The cellular effects of plasma-generated reactive species in various environments have been poorly understood. Thus, in this study we elucidated the environment’s impact on the antimicrobial activity of plasma, particularly focusing on the nature of surrounding fluids using a model filamentous fungus, Neurospora crassa (bread mold). Our data revealed that the ionic strength of background solutions modulated the antimicrobial efficiency of plasma, regardless of the quantity of reactive species.

Materials and methods

Fungus and treatment with plasma

N. crassa (wild-type strain; ORS-SL6a), known as bread mold, was used in the study. N. crassa was grown and kept in Vogel's minimal medium at 30 °C in the dark for 2 days and then at 25 °C in the light for at least 3 days. Fungal spores were harvested from a 2-week-old culture and used for plasma application.

The nonthermal plasma jet was generated at atmospheric pressure using argon (Ar) as the fed gas and was used for treating fungal spores as previously described (Fig. 1A) [29]. Fungal spores (2 × 10⁷ spores) seeded in 1 ml of sterile water, saline (145 mM NaCl), or other indicated solutions were placed in a 48-well microtiter plate. The Ar plasma jet was applied to each well for the indicated time. The distance between the tip of needle (inner electrode) and the microtiter plate surface was 1.7 cm and the conditions for plasma generation were 4 kV voltage, 13 mA current, 22 kHz repetition rate, and 0.4 L/min Ar gas flow. For the control, only Ar gas without plasma discharge was applied to samples.

Test for spore germination and structure

N. crassa spores harvested from a 2-week-old culture were suspended in sterile water, saline, or other indicated solutions and exposed to Ar plasma jet. Treated spores (2 × 10⁷) were washed with 1 ml of corresponding solution and resuspended in new solution. After serial dilution, 100 μl of diluted suspension was spread onto a fructose–glucose–sorbose agar plate and the number of colonies, which indicated germinated spores, was counted after incubation at 30 °C for 2 days.

The structure of spores after plasma treatment was analyzed by transmission electron microscopy (TEM). Spores treated with Ar gas (control) and plasma in water and saline for 3 min were used for the analysis. The specimen preparation for TEM analysis was carried out as described previously [23]. Spores exposed to plasma or Ar gas were fixed in 1 ml of Karnovsky’s fixative (2% paraformaldehyde and 2% glutaraldehyde) at 4 °C overnight. After three washes, the spores were fixed again in 1 ml of 1% osmium tetroxide (in 1/2 phosphate-buffered saline) at room temperature for 2 h. Subsequently, dehydration was performed by incubating the spores for 10 min sequentially in 1 ml of 30, 50, 70, 80, 90, and 100% (three times) ethanol. Dehydrated spores were treated with 1 ml of 100% propylene oxide twice at room temperature for 15 min and embedded into resin as described previously [23]. The polymerized resin block (containing spores) was microsectioned,
and the sectioned specimen was examined under TEM (JEM 1010, JEOL, Tokyo, Japan).

### Assay for genomic DNA oxidation

Fungal spores treated with Ar gas (control) and plasma for 3 min were collected and vacuum-dried. Dried spores were macerated to powder in liquid nitrogen, and genomic DNA was extracted using a Gentra Puregene DNA extraction kit (Qiagen, Germantown, MD, USA) following the manufacturer’s protocol. About 1 μg of DNA was incubated at 95 °C for 5 min and then quickly moved to ice to generate single strands. Single-stranded DNA was digested to nucleosides after incubation with nuclease P1 at 37 °C for 2 h, followed by alkaline phosphatase treatment. After centrifugation (8000 rpm for 5 min), the assay for 8-OHdG (8-hydroxydeoxyguanosine), a product of DNA oxidation, in the supernatant was measured using a portable pH meter (Eutech Instruments, Singapore) as described previously [23]. Reactive species (hydroxyl radical, nitric oxide, peroxynitrite) in solutions were measured using tert-butylhydroperoxide (90 μM final concentration) as an electron source. The oxidative activity of the solutions was expressed as the rate of hydrolysis of the substrate. The CD spectrum in the 200 and 350 nm wavelength range was measured using a CD spectrophotometer (HR400, Ocean Optics, Dunedin, FL, USA) and a UV–visible spectrophotometer (S-3100, Scinco, Seoul, Korea), respectively. For emission spectroscopy, 1 ml of each solution was placed in a quartz cuvette and a plasma jet was applied to the solution during plasma exposure, the intensity of the light emitted from the solutions was recorded over wavelength. For absorption spectroscopy, 1 ml of each solution was treated with Ar gas (control) or plasma for 3 min, and the absorption of solutions was immediately monitored over wavelengths. The CD spectra of spores were analyzed by using a CD spectrophotometer (J-815, Jasco, Easton, MD, USA). Fungal spores (2 × 10^7) seeded in 1 ml of water and saline were exposed to Ar gas (control) and plasma for 3 min. Spores were pelleted by centrifugation (10,000 rpm for 5 min), and the supernatant was removed. The pellets were resuspended in 1 ml of water or saline and used for measuring CD spectra.

After plasma treatment, emission and absorption spectra were analyzed in water and other solutions by using a CCD spectrometer (HR400, Ocean Optics, Dunedin, FL, USA) and a UV–visible spectrophotometer (S-3100, Scinco, Seoul, Korea), respectively. For emission spectroscopy, 1 ml of each solution was placed in a quartz cuvette and a plasma jet was applied to the solution. During plasma exposure, the intensity of the light emitted from the solutions was recorded over wavelength. For absorption spectroscopy, 1 ml of each solution was treated with Ar gas (control) or plasma for 3 min, and the absorption of solutions was immediately monitored over wavelengths.

### Measurement of membrane depolarization

Membrane depolarization was measured by using the FIVE-photon membrane potential assay kit (FIVEPhoton Biomedicals, San Diego, CA, USA), following the manufacturer’s protocol. Fungal spores (2 × 10^7) were treated with Ar gas (control) or plasma in various solutions for 3 min and washed with distilled water. After centrifugation (10,000 rpm for 5 min), the spores were resuspended in 100 μl of 1 × external assay buffer and 100 μl of 2 × loading dye. Spores with dye were incubated at 37 °C for 15 min and transferred to a 96-well plate. The plate was read at 530/565 nm using a microplate reader (BioTek).

### Results

#### NaCl level in the background solution can attenuate the harmful effects of plasma

N. crassa spores submerged in water or saline (145 mM NaCl) were exposed to nonthermal atmospheric pressure plasma as shown in Fig. 1A. Fungal spores exhibited more damage to germination, morphology, structure, and molecules when treated in water than in saline. Spore germination in water was dramatically reduced after plasma treatment in a time-dependent manner, whereas no significant reduction in germination was observed after a 3-min saline treatment (Fig. 1B). Furthermore, the majority of the plasma-treated spores were crushed and internally less dense in water compared to the Ar-gas-treated control (Fig. 1C). In saline, no significant difference was observed in the shape and internal structure between spores treated with plasma and those treated with Ar gas (Fig. 1C).

Oxidation of genomic DNA, assessed by the amount of 8-OHdG (product of DNA oxidation), was increased in spores treated with plasma in water compared to those treated in saline (Fig. 2A). The CD spectrum in the 200 and 350 nm wavelength range was significantly different for the spores treated with plasma in water compared to the control (Ar-gas-treated spores; Fig. 2B), which implies alterations in molecular structure. In contrast, no difference was observed in spores treated in saline compared to control (Fig. 2B). Spores in water became paler after plasma treatment, whereas those in saline exhibited slightly less orange color (Fig. 2C). The orange pigmentation of spores results from the accumulation of neurosporaxanthin and precursor carotenoids [32]. Thus, this result suggests that pigments may be destroyed after plasma treatment in water.

When fungal spores were exposed to plasma in various concentrations of NaCl solution, we observed that spore germination increased in higher NaCl concentrations. The majority of spores germinated in solutions with a NaCl concentration greater than 14.5 mM (Fig. 3A). After plasma treatment, the spore germination rate decreased in NaCl above 290 mM (Fig. 3A), which may be due to the additional effect of salt stress.

From these results, it can be suggested that the presence of NaCl (greater than 14.5 mM) in the background fluid can provide protective effects on germination, structure, and genomic DNA of fungal spores against plasma toxicity.

#### pH and chemical changes in NaCl solution do not affect spore germination

Our data clearly show that environmental NaCl modulates plasma’s antimicrobial activity. To clarify the mechanism for this modulation, we examined plasma-induced changes in NaCl solution, such as pH and chemistry. The pH of water and NaCl solutions decreased to between 3 and 4 after plasma treatment.
for 3 min (Fig. 3B). We made a similar observation during Ar plasma treatment in the previous study [23]. This indicates that many spores in NaCl solution are still able to germinate in the acidic pH caused by plasma.

The absorption spectra of plasma-treated water and saline (145 mM NaCl) showed that peaks between 210 and 250 nm were generated by plasma treatment in both solutions (Fig. 3C). Although not all peaks were chemically identified, no significant
Fig. 3. Effects of NaCl during plasma treatment. (A) Germination of spores treated with various concentrations of NaCl. Spores were treated with plasma for 3 min. (B) pH of NaCl solutions treated with plasma. In (A) and (B), each value represents the average of three replicate measurements. (C) Absorption spectra measured in water and saline (145 mM NaCl). (D) Spore germination after direct treatment with plasma (top) or incubation in plasma-treated solutions (bottom). For the plasma-treated solutions, water and saline were treated with plasma for 3 and 5 min. Then, spores were incubated in those treated solutions for 3 and 5 min. The relative germination (%) indicated on the graph was calculated as follows: (number of germinated spores treated with plasma/number of germinated spores treated with Ar gas) x 100. Each value represents the average of three replicate measurements. **p < 0.01; Student’s t test.

Fig. 4. Effects of sorbitol during plasma treatment. (A) Germination of spores after plasma treatment for 3 min. (B) pH of sorbitol solutions after plasma treatment for 3 min. Each value represents the average of three replicate measurements.
difference in the absorption spectra was observed between water and saline (Fig. 3C). Finally, incubation of spores in the plasma-treated water and saline for the same period of time as used in the direct plasma treatment did not show any difference in germination (Fig. 3D). Because the protective effect of NaCl is observed during direct plasma treatment (Fig. 3D), it suggests that the interaction with plasma influences fungal spores more than the property of solutions after plasma treatment.

Because NaCl in solution provides osmotic stability to fungal spores, we used a known osmotic stabilizer, sorbitol, to test if osmotic stabilization modifies the effects of plasma. When spores in various concentrations of sorbitol were treated with plasma for 3 min, we observed that spore germination in all concentrations was as low as in water (Fig. 4A). Spores treated in 1 M sorbitol (concentration known to provide osmotic stability to protoplasts) germinated more often compared to those in lower concentrations, probably because of the osmotic protection provided by sorbitol (Fig. 4A). Although we observed this recovery of germination in 1 M sorbitol, the germination rate was still lower than that observed in saline (145 mM NaCl). As shown in NaCl solutions, plasma also lowered the pH of sorbitol after 3 min treatment (Fig. 4B).

Our data demonstrated that the reduced antifungal effects of plasma observed in NaCl solution were not exclusively related to pH, chemical alteration, or osmotic balance.

The amount of reactive species is not associated with fungal inactivation

The different strengths of the antimicrobial effects observed in water, saline, and sorbitol may be associated with the amount of reactive species that are generated in solutions during plasma

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**Fig. 5.** The concentration of reactive species in solutions. (A) Emission spectra of water, NaCl, and sorbitol solutions during plasma treatment. (B) Spectrum area showing hydroxyl radical (·OH), hydrogen peroxide (H₂O₂), and singlet oxygen (¹O₂) peaks. (C) Level of reactive oxygen and nitrogen species measured in solutions after plasma treatment for 3 min using fluorescent indicators, H₂DCF (major species: hydroxyl radical and peroxynitrite), DAF-FM (major species: nitric oxide), and TA (major species: hydroxyl radical). The relative fluorescence measured as the ratio of fluorescence units between Ar gas (control) and plasma-treated solutions is indicated on the graph: fluorescence units of plasma treated solution/fluorescence units of Ar-gas-treated solution. Each value on the graph represents the average of 9–12 replicates from three or four replicate experiments (for H₂DCF and DAF-FM) or three replicates from an experiment (for TA). The p values for comparison between water and other solutions, and between saline and other solutions, were less than 0.01 except for DAF-FM fluorescence between water and saline (Student’s t test).
treatment, because various reactive species are generated inside solutions when they are exposed to plasma [31]. Using emission spectroscopy analysis, we observed the generation of reactive oxygen species such as hydroxyl radical (\(^{\cdot}\text{OH}\)), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), and singlet oxygen (\(\text{^1O}_2\)) when water, saline, and sorbitol were exposed to plasma (Fig. 5A). We also observed peaks around 700–900 nm, which were mostly neutral excited species of Ar gas.

After plasma treatment for 3 min, the amount of reactive species in the solutions was generally higher in water than in saline and sorbitol (Fig. 5C). Saline (145 mM NaCl) showed a slightly lower level of reactive species than water and a significantly greater level than sorbitol (Fig. 5C). In two concentrations of sorbitol, 145 mM (the same concentration as NaCl in saline) and 1 M (concentration imparting osmotic stability to protoplast), no or low abundance of reactive species was detected after plasma exposure (Fig. 5C).

Although the level of reactive species in NaCl solution was similar or slightly lower than that in water during plasma treatment, it was significantly higher than that in sorbitol, indicating that the higher level of fungal inactivation observed in water and sorbitol by plasma could not be completely explained by the amount of reactive species.

**Ionic strength of solutions modulates the plasma activity**

The concentration of reactive species measured in our analysis may influence the level of spore inactivation, but it does not explain why, in the presence of higher concentrations of reactive species, fungal spores were more protected in NaCl than in sorbitol. To answer this question, we analyzed the spore germination after adding NaCl to sorbitol. When spores in sorbitol solutions were exposed to plasma with the addition of NaCl (145 mM), the germination rate was significantly increased and similar to that observed in saline (Fig. 6A). The recovery of the germination rate by the addition of NaCl was observed at two sorbitol concentrations (0.5 and 1 M; Fig. 6A). This observation demonstrates that NaCl itself may be responsible for modulating the antimicrobial activity of plasma.

To examine this hypothesis, we treated spores in other ionic solutions (KCl and CaCl\(_2\)) with the same concentration tested for NaCl (145 mM) with plasma. The plasma treatment did not significantly change the germination rate from the control in the case of spores in ionic solutions, whereas more than 50% of spores in water or nonionic solutions, sorbitol, glycerol, and sucrose (145 mM), were inactivated after plasma treatment (Fig. 3A). The peak levels of hydroxyl radical (309 nm), hydrogen peroxide (307 nm), and singlet oxygen (777.53 nm) were reduced in saline and sorbitol compared to water (Fig. 5B). When the NaCl concentration in solution was increased, the levels of hydroxyl radical and hydrogen peroxide slightly decreased (Fig. 5B, middle).

Although the level of reactive species in NaCl solution was similar or slightly lower than that in water during plasma treatment, it was significantly higher than that in sorbitol, indicating that the higher level of fungal inactivation observed in water and sorbitol by plasma could not be completely explained by the amount of reactive species.
The dynamics of reactive species generated by plasma may be affected by the interaction with ions. This observation provides an insight into the behaviors of reactive species in biological environments that may have various ion strengths. Another implication is that antimicrobial control strategies that use reactive species may result in a variety of outcomes depending on the level of ions in the environment. The current demand for nonthermal and environmentally friendly sterilization tools without microbial resistance makes nonthermal plasma at atmospheric pressure a promising means of microbial inactivation. Our study suggests that the antimicrobial activity of plasma can be controlled (elevated or attenuated) by managing the microenvironment surrounding microbes.

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