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Original Contribution

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

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ABSTRACT

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.

The antimicrobial activity of reactive species has been applied to developing disease control strategies (for review see [1]). Antibiotics such as aminoglycosides, quinolines, rifampicin, and chloramphenicol can metabolically induce intracellular reactive oxygen species (ROS) production, killing microorganisms [2–5]. Photosensitizers and photocatalytic nanoparticles have been shown to inactivate microbes by generating ROS in response to certain wavelengths of light [6]. These tools are effective in microbial inactivation but their widespread application has been limited because reactive species are likely to damage both microorganisms and hosts. However, reactive species have recently been shown to have a broad spectrum of biological functions (from harmful to beneficial) depending on their concentration, the exposed organism's species, and the surrounding environment [7–9].

The functional diversity of reactive species may explain the various cellular effects of atmospheric-pressure nonthermal plasma, which is an antimicrobial tool producing reactive species. Plasma is known to be the fourth state of matter and is generated by applying high energy (electric voltage) to gas at atmospheric pressure, leading to the formation of various reactive species, UV light (>300 nm), and electrons [10]. Plasma can not only inactivate microorganisms and cancer cells but also promote wound healing and regeneration [11]. The antimicrobial efficiency of plasma can be modulated by altering the physical conditions of plasma discharge such as electrical power, electric pulse, fed gases, and exposure time [12–14]. Different microbes, especially prokaryotes and eukaryotes, exhibit different levels of susceptibility to plasma [15–21]. Furthermore, microorganisms in various physiological fluids are inactivated differently by plasma [22,23], and plasma is more effective at eradicating bacteria on nonbiological

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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 (2×10^7 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 (2×10^7) 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 × 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,

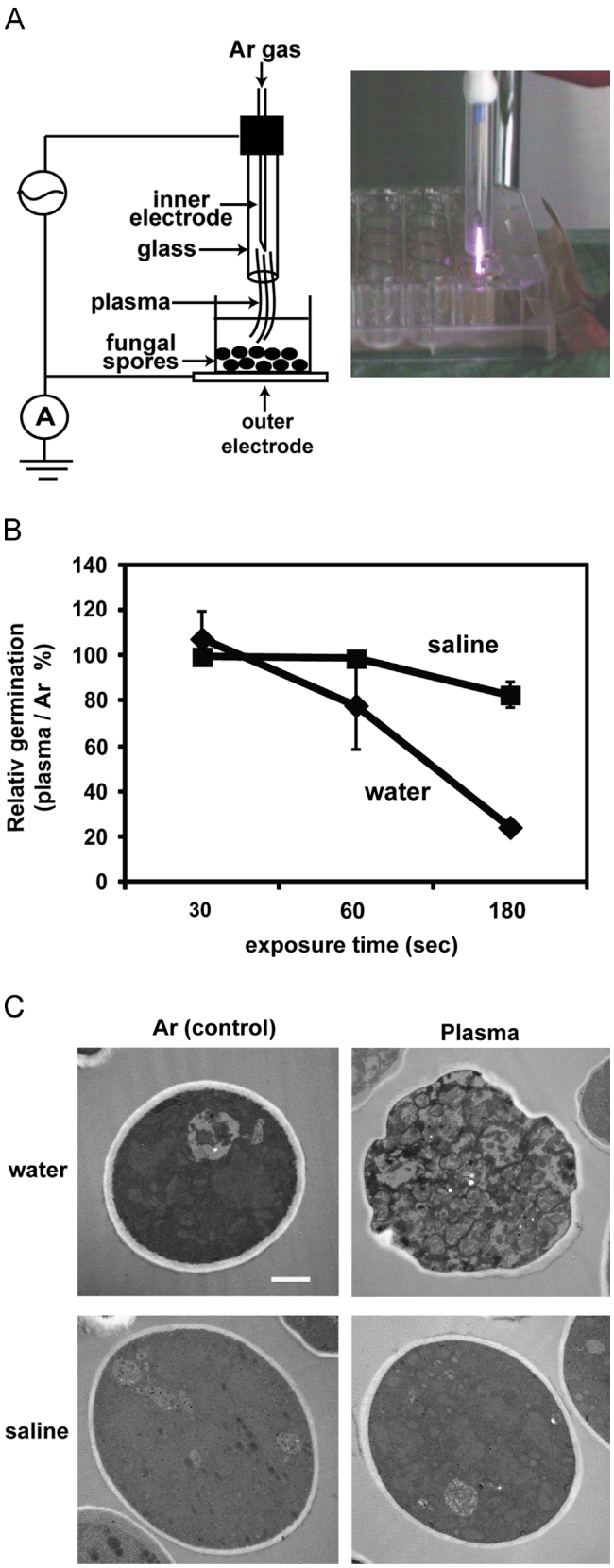

Fig. 1. Spore germination and structure after plasma treatment. (A) Schematic view (left) and picture (right) of sample treatment with Ar plasma jet. (B) Germination and (C) internal structure of *N. crassa* spores after plasma treatment. The relative germination (%) indicated on the graph was calculated as follows: (number of germinated spores treated with plasma/number of germinated spores treated with Ar gas) × 100. Each value represents the average of three replicate measurements and Student's *t* test was performed; $p < 0.01$ at 180 s exposure. Scale bar, 1 μm.