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Sterilization of Bacteria, Yeast, and Bacterial Endospores by Atmospheric-Pressure Cold Plasma using Helium and Oxygen

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Atmospheric-pressure cold plasma (APCP) using helium/oxygen was developed and tested as a suitable sterilization method in a clinical environment. The sterilizing effect of this method is not due to UV light, which is known to be the major sterilization factor of APCP, but instead results from the action of reactive oxygen radicals. *Escherichia coli*, *Staphylococcus aureus*, and *Saccharomyces cerevisiae* deposited on a nitrocellulose filter membrane or *Bacillus subtilis* spores deposited on polypropylene plates were exposed to helium/oxygen plasma generated with AC input power at 10 kHz, 6 kV. After plasma treatment, nitrocellulose filter membranes were overlaid on fresh solid media and CFUs were counted after incubation overnight. D-values were 18 sec for *E. coli*, 19 sec for *S. aureus*, 1 min 55 sec for *S. cerevisiae*, and 14 min for *B. subtilis* spores. D-values of bacteria and yeast were dependent on the initial inoculation concentration, while the D-value of *B. subtilis* spores showed no correlation. When treated cells were observed with a scanning electron microscope, *E. coli* was more heavily damaged than *S. aureus*, *S. cerevisiae* exhibited peeling, and *B. subtilis* spores exhibited shrunken morphology. Results showed that APCP using helium/oxygen has many advantages as a sterilization method, especially in a clinical environment with conditions such as stable temperature, unlimited sample size, and no harmful gas production.

Keywords: sterilization, atmospheric-pressure cold plasma, *Escherichia coli*, *Staphylococcus aureus*, *Saccharomyces cerevisiae*, *Bacillus subtilis*

There has been a continued demand for novel sterilization techniques appropriate for use on various clinical devices. Conventional sterilization technologies, such as autoclaves, ovens, and chemicals like ethylene oxide, rely on irreversible metabolic inactivation or breakdown of vital structural components of microorganisms. Because heat and steam are not suitable for use on heat-sensitive materials, hydrogen peroxide and ethylene oxide are commonly used as low-temperature sterilization techniques for these devices. However, the carcinogenic property of ethylene oxide residues requires that the time needed for complete ventilation be longer than the actual time taken to sterilize the materials (Holyoak *et al.*, 1996; Lucas *et al.*, 2003). Another interesting sterilization technology is gamma irradiation for heat-sensitive materials, but it is costly and its safe operation requires an isolated site (Affatato *et al.*, 2002). The various limitations of the existing sterilization techniques have required the development of alternative techniques that are cheap, effective, and do not generate toxic residues. Recently, irradiation with electron beams (Lara *et al.*, 2002), high electric field (Uemura and Isobe, 2002), high-voltage pulse (Oshima and Sato, 2002), pulsed light irradiation (Takeshita *et al.*, 2003), and negative and positive ions (Noyce and Hughes, 2002) have been applied as sterilization systems. However, the efficiencies of these new sterilization systems were guaranteed only at high treatment temperatures.

In addition to these sterilization systems, substantial amounts of research have been focused on sterilization using plasma in recent years. Plasma is a partially ionized, low-pressure gas that is comprised of ions, electrons, and ultraviolet (UV) photons, as well as reactive neutral species (radicals and excited atoms and molecules) with sufficient energy to break covalent bonds and initiate various chemical reactions (Moisan *et al.*, 2002). During plasma treatment,

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microorganisms are sterilized as a result of direct contact with ions of high kinetic energy and electrons, as well as UV rays. Even though UV light is the major sterilization factor of plasma, it is limited due to the lack of penetration and strong dependence on the distance from the UV source, which may result in non-homogeneous microbial sterilization. This is because UV irradiation does not penetrate most materials and significant amounts of irradiation are absorbed by glass and plastics. Therefore, sterilization of microorganisms on surfaces by UV irradiation is strongly dependent on the structure of the surface. The main risk associated with plasma treatment is the extreme rise of temperature that results from the increase in power density.

The objective of this study was to develop an atmospheric-pressure cold plasma (APCP) system that uses helium/oxygen (He/O_2) and is able to effectively penetrate into complicated structures such as tubes and artificial organs without producing harmful residues or increasing temperature. To accomplish this, helium gas was chosen because of its lack of chemical activity and its lack of specific effects on microorganisms despite the intense ion and electron bombardments; oxygen gas was used to increase the sterilizing effect.

Materials and Methods

Apparatus

A plasma sterilization system was constructed as shown in Fig. 1. This system uses a 10~20 kHz and 4~20 kV generator to excite helium (He) and oxygen (O_2) gas injected from a gas container into a plasma generator (400 × 500 × 3 mm for bacteria and yeast, and 200 × 200 × 3 mm for bacterial spores) at a constant flow rate. The gas was excited into the plasma state at 10 kHz frequency and 6 kV power energy input by passing through a pair of He/O_2 plasma generators, where a filter or carrier was positioned between the two plasma generators. The distance between the two plasma generators was 6 mm.

Preparation of microorganisms

Microorganisms used in the current study were *Escherichia coli* ATCC 25922 (Gram-negative bacteria), *Staphylococcus aureus* ATCC 29213 (Gram-positive bacteria), and *Saccharomyces cerevisiae* KCTC 7915 (yeast). *E. coli* and *S. aureus* were grown on brain heart infusion medium (BHI, Difco, USA) at 37°C. *S. cerevisiae* was grown on yeast peptone dextrose medium (YPD, Difco) at 25°C. The overnight culture (500 μl) was transferred to 5 ml of appropriate fresh medium and incubated with shaking until the optical density at 600 nm reached 0.5. Cells were then serially diluted in saline to concentrations of 10^2 to 10^6 CFU/ml. One ml of each diluted sample was filtered through a 0.45 μm nitrocellulose membrane filter (25 mm diameter, Millipore, USA) using a vaccum manifolder (BioRad, USA), and filtrate was dried in the air.

Preparation of spores

Bacillus subtilis ATCC 6633 was grown on nutrient agar (NA, Difco) at 37°C for 5 days in order to

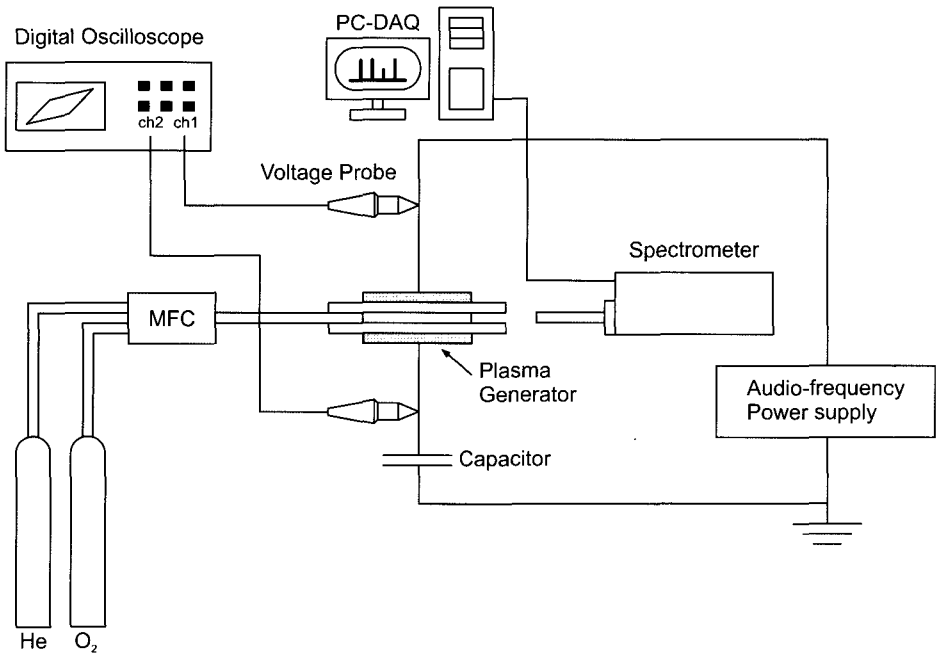

Fig. 1. Schematic diagram of an atmospheric pressure cold plasma generator using He/O_2 and a diagnostic system.

produce spores. Bacterial cells were harvested by scraping and were suspended in distilled water. Vegetative cells were lysed with lysozyme (200 μg/ml, Sigma, USA) at 37°C for 1 h. After washing with a buffer (17.3 mM SDS, 1 M NaCl, 1 mM NaCl) and then with distilled water using centrifugation, spores were suspended in saline and kept at −18°C until use. Spore solution was serially diluted in saline to concentrations of 10^7 to 10^{10} spores/ml, and 10 μl of each solution was loaded on a polypropylene carrier [15 mm (width) × 15 mm (length) × 1 mm (height)] and dried in the air.

Plasma treatment

Microorganisms on a filter or *B. subtilis* spores on a polypropylene carrier were exposed to He/O_2 plasma for various time periods. Samples that had not been exposed to plasma were used as controls and kept at room temperature while samples were treated. After treatment, each filter was overlaid on fresh MacConkey (Difco) agar for *E. coli*, BHI agar for *S. aureus*, or YPD agar for *S. cerevisiae* and incubated at 37°C for *E. coli* and *S. aureus* or 25°C for *S. cerevisiae*. The number of colonies appearing on each filter was counted after overnight incubation. In the case of the *B. subtilis* spores, polypropylene carriers with *B. subtilis* spores were soaked in 10 ml saline for 10 min with shaking to detach spores. The numbers of *B. subtilis* spores in 100 μl were assayed using a pouring method.

Calculation of D-value

The death-value (D-value) was identified as the time required to achieve 10% reduction of the initial numbers at the specific plasma treatment condition. The D-value for each microorganism was obtained by averaging the numbers obtained at various initial concentrations of cells.

Scanning electron microscopy

Microorganisms were fixed with 2.5% glutaraldehyde for 2 h and then dehydrated in a graded series of ethanol. After samples were coated with platinum in a sputter coater, they were examined with an SEM (JSM-840A, JEOL, Japan).

Results

Optical emission spectrum of He/O_2 plasma

Optical emission spectrum (OES) of the He/O_2 plasma produced peaks at 706 nm for the helium atom and 777 nm for the oxygen radical, while no peak was produced at 220-280 nm, to the normal spectrum of UV irradiation (Fig. 2).

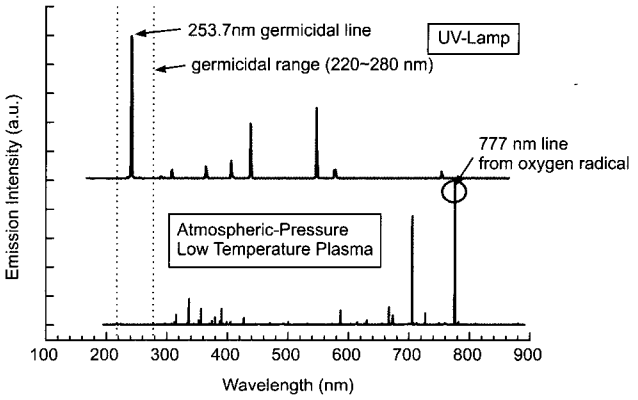

Fig. 2. Optical emission spectra of a conventional ultra-violet lamp (above) and the He/O_2 plasma (below).

Sterilizing effect of APCP using He/O_2 on E. coli

The sterilizing effect of APCP using He/O_2 on *E. coli* was higher during the early treatment period than during the later treatment period (Fig. 3A). Complete sterilization was accomplished within 30 sec at 10^2 CFU/filter, while longer than 120 sec was necessary to completely sterilize 10^5 CFU/filter.

Sterilizing effect of APCP using He/O_2 on S. aureus

The sterilizing effect of APCP using He/O_2 on *S. aureus* was similar to that of *E. coli*, except a longer treatment time was needed for complete sterilization (Fig. 3B). *S. aureus* was completely sterilized within 45 sec, while complete sterilization at 10^5 CFU/filter was achieved after 90 sec.

Sterilizing effect of APCP using He/O_2 on S. cerevisiae

S. cerevisiae was more resistant against the plasma treatment than *E. coli* or *S. aureus* (Fig. 3C). Even at the initial inoculation density of 10^1 CFU/filter, it took 3 min for complete sterilization, 6 times longer than needed that for *E. coli*.

Sterilizing effect of APCP on B. subtilis spores

A time interval of 10 min was chosen for spores because of their resistance to plasma. Different results were obtained in *B. subtilis* spores compared to bacteria (Fig. 3D). Biphasic curves were observed with an initial rapid death phase within 10 min, followed by tailing without complete sterilization even after treatment for 60 min. The initial death value decreased as the initial density decreased. Even at the lowest initial inoculation size (10^4 CFU/filter), complete sterilization was not observed within 60 min.

Sterilizing effect of APCP on B. subtilis spores at different densities

The potential effect of density on the sterilization of

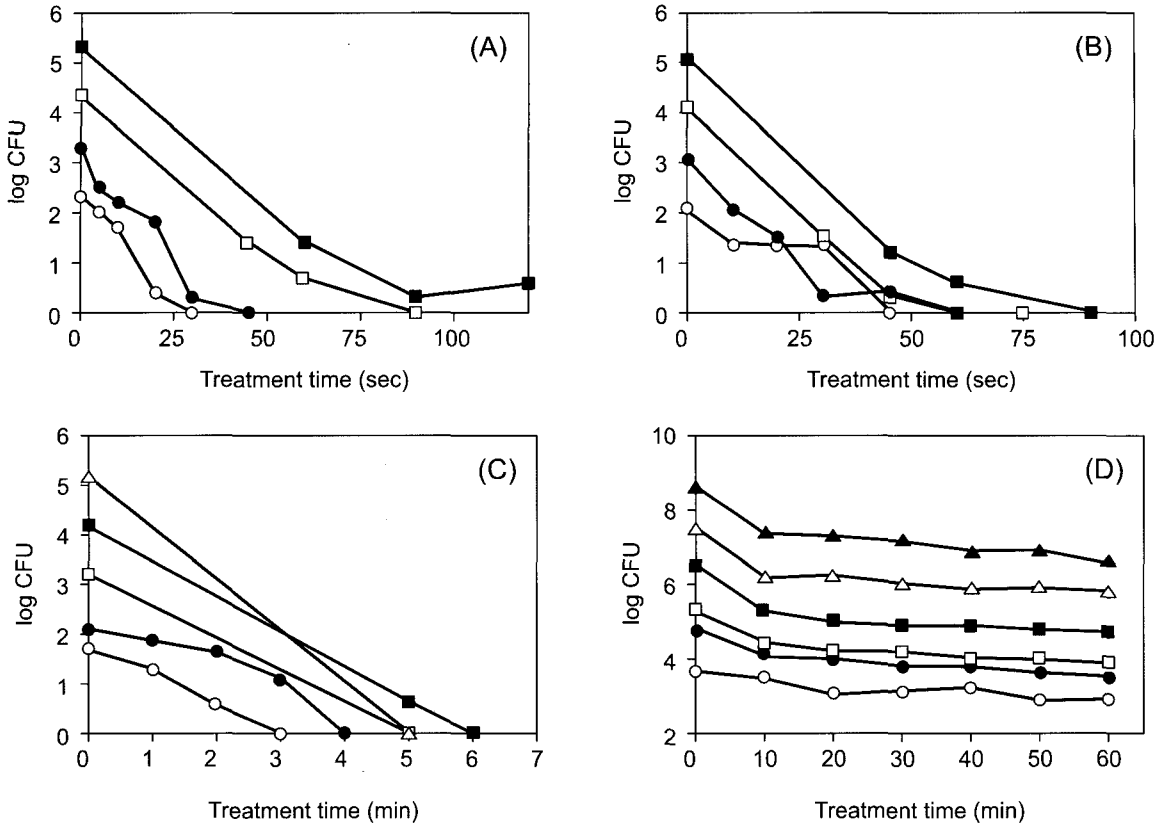

Fig. 3. Sterilization of various microorganisms with atmospheric pressure cold plasma using He/O_2. (A) *Escherichia coli* (○, 10^2 CFU; ●, 10^3 CFU; □, 10^4 CFU; ■, 10^5 CFU); (B) *Staphylococcus aureus* (○, 10^2 CFU; ●, 10^3 CFU; □, 10^4 CFU; ■, 10^5 CFU; (C) *Saccharomyces cerevisiae* (○, 10^1 CFU; ●, 10^2 CFU; □, 10^3 CFU; ■, 10^4 CFU; △, 10^5 CFU); (D) *Bacillus subtilis* spore (○, 10^3 CFU; ●, 10^4 CFU; □, 10^5 CFU; ■, 10^6 CFU; △, 10^7 CFU; ▲, 10^8 CFU)

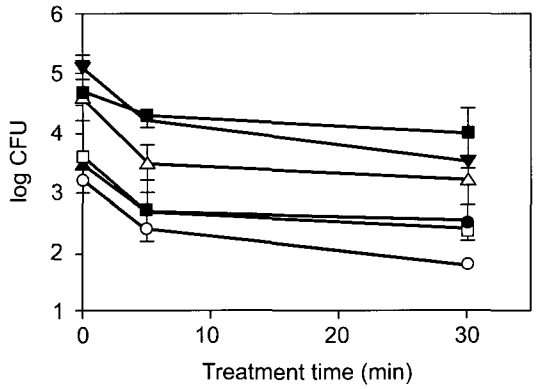

Fig. 4. Sterilization of *B. subtilis* spores at various densities with atmospheric pressure cold plasma using He/O_2. ○, 10^5 CFU across a small area; ●, 10^5 CFU across a mid-sized area; □, 10^5 CFU across a wide area; ■, 10^7 CFU across a small area; △, 10^7 CFU across a mid-sized area; ▲, 10^7 CFU across a wide area.

B. subtilis spores was tested. The same numbers of *B. subtilis* spores diluted in different volumes of water were attached to carriers in order to vary the surface areas exposed to the plasma. Results showed no difference in sterilization effect from the exposed surface area to the plasma (Fig. 4).

D-value

D-values were obtained from more than three independent experiments using various initial concentrations as shown in Table 1. The D-values at the initial concentrations were 18 sec for *E. coli*, 19 sec for *S. aureus*, 1 min 45 sec for *S. cerevisiae*, and 14 min for *B. subtilis* spores.

Scanning electron microscopy

Images from a scanning electron microscope (SEM) of bacteria, yeast, and *B. subtilis* spores before and after plasma treatment are presented in Fig. 5. All tested microorganisms exhibited significant damage on the cell wall to various degrees. *E. coli* was the most heavily damaged by plasma treatment. On the other hand, *S. cerevisiae* exhibited a limited morphological change. A phenomenon that appeared much like peeling was observed on the external surfaces of bacteria and yeast. In the case of *B. subtilis* spores, shrunken morphology was shown.

Table 1. D-values of tested microorganisms

Initial CFU	*E. coli*	*S. aureus*	*S. cerevisiae*	*B. subtilis*
10^1	-[1]	-	3 min	-
10^2	15 sec	23 sec	2 min	-
10^3	15 sec	20 sec	1 min 40 sec	-
10^4	23 sec	15 sec	1 min 30 sec	30 min
10^5	-[2]	18 sec	1 min 24 sec	10 min
10^6	-	-	-	10 min
10^7	-	-	-	10 min
10^8	-	-	-	10 min
Average D-value	18 sec	19 sec	1 min 55 sec	14 min

[1], not tested; [2], Too many to count

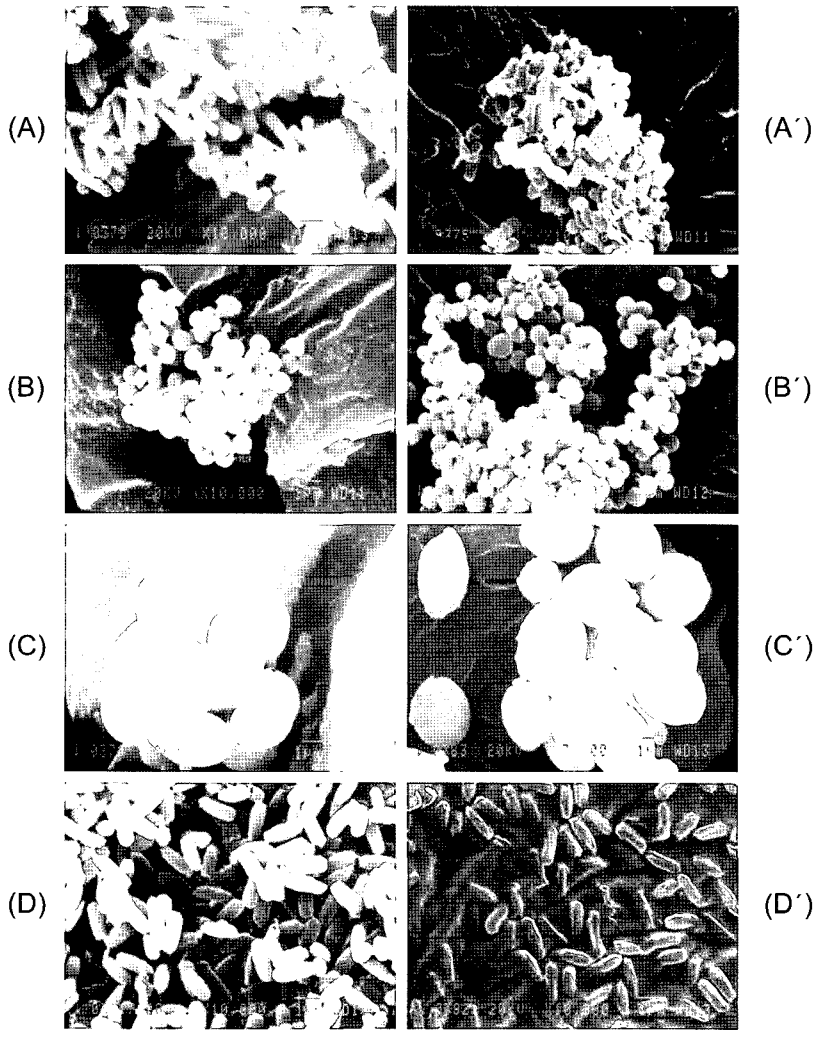

Fig. 5. Scanning electron micrographs of various microorganisms with atmospheric pressure cold plasma using He/O_2. (A, untreated *E. coli*; B, untreated *S. aureus*; C, untreated *S. cerevisiae*; D, untreated *B. subtilis* spore; A′, plasma-treated *E. coli*; B′, plasma-treated *S. aureus*; C′, plasma-treated *S. cerevisiae;* D′, plasma-treated *B. subtilis* spore).

Discussion

This study showed that APCP using He/O_2 generates a low level of UV while producing reactive oxygen radicals that likely act as the main factor for sterilization; these reactive oxygen radicals carry the advantage of being capable of accessing the interiors of complicated structures such as tubes and artificial organs. Moreover, this new system did not produce excessive heat when used for up to 1 h of treatment. Different microorganisms showed different sensitivities to He/O_2 plasma, and the order of sensitivity coincided with the structrue of the cell wall. The fact that *S. aureus* had a higher D-value than did of *E. coli* suggests that peptidoglycan works as a physical shield against plasma. This was also observed with *S. cerevisiae*, the cell wall structures of which differ from those of bacteria and more difficult to mechanically rupture. *B. subtilis* spores showed extreme resistance to plasma. In the case of *B. subtilis* spores, similar shrunken forms have been observed upon treatment with electron beams or electric fields (Hury *et al.*, 1998; Lara *et al.*, 2002; Park *et al.*, 2004). This finding supported the aforementioned hypothesis, which proposed that He/O_2 plasma showed a sterilizing effect via oxygen radicals that can access and directly attack the cell wall. Further study is needed to determine how oxygen radicals can peel off the bacterial cell wall or shrink spores, resulting in the death of the microorganisms.

For sterilization of *B. subtilis* spores, we came to the following conclusions: first, sterilization efficiency was not affected by the initial spore density. This was similar to another report (Lerouge *et al.*, 2000b) that showed similar sterilization patterns regardless of different initial spore densities. Second, complete sterilization at high density could not be achieved even after 120 min of treatment (data not shown) as others reported (Spilimbergo *et al.*, 2003). Third, the sterilization kinetics of *B. subtilis* spores by APCP using He/O_2 showed a biphasic curve with a faster decrease in spore viability at the earliest stage, followed by a slower decrease at later stages. Similar biphasic sterilization phenomena have been reported by others (Lerouge *et al.*, 2000a; Lucas *et al.*, 2003; Oshima and Sato, 2004). They explained this to result from unequal distribution of lethal events taking place during plasma treatment. This means that a portion or a layer of the spore population was inactivated at a specific time.

We suggest that this phenomenon is related to the direct contact of oxygen radicals to the surfaces of microbes; this contact acts to sterilize, but holds limited ability to penetrate the surfaces. If this were true, the increase in the number of the exposed spores resulting from dilution would increase the sensitivity of spores to plasma. In this study, this was observed during the first 5 min at every concentration. However, there was no difference in sensitivity after 30 min treatment, and we were not able to completely sterilize *B. subtilis* spores. At least 1% of all *B. subtilis* spores were extremely resistant to plasma at various concentrations and densities. This may be a product of the heterogeneous *B. subtilis* population or for other reasons that have not yet been elucidated.

In conclusion, APCP using He/O_2 can efficiently sterilize various microorganisms, including *B. subtilis* spores, without producing any harmful residues. It requires a short treatment time (less than 20 sec for bacteria, less than 2 min for yeast, and 14 min for bacterial spores) and does not expose materials to high temperatures or pressures, both of which are produced from classical sterilization methods such as autoclaving, chemical treatment, and high doses of irradiation. Unlike UV light, oxygen radicals produced by APCP using He/O_2 can access and kill bacteria contaminating outside surfaces, and can also sterilize the inside surfaces of various apparatuses. These characteristics suggest APCP using He/O_2 as a candidate for an efficient sterilization system for a variety of equipment used in a clinical environment.

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