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Decomposition of Biological Macromolecules by Plasma Generated with Helium and Oxygen

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In this study, we attempted to characterize the biomolecular effects of an atmospheric-pressure cold plasma (APCP) system which utilizes helium/oxygen (He/O_2). APCP using He/O_2 generates a low level of UV while generating reactive oxygen radicals which probably serve as the primary factor in sterilization; these reactive oxygen radicals have the advantage of being capable to access the interiors of the structures of microbial cells. The damaging effects of plasma exposure on polypeptides, DNA, and enzyme proteins in the cell were assessed usiing biochemical methods.

Keywords: sterilization, atmospheric-pressure cold plasma, reactive oxygen species, biological macromolecules destruction

In the medical, food processing, heating, ventilation, and air conditioning industries, a constant search is being undertaken for improved pasteurization, disinfection, and sterilization technologies. Candidate techniques must address and overcome problems including thermal sensitivity and destruction by heat, the formation of toxic by-products, high costs, and operational inefficiency concerns. Sterilization is a physical or chemical act or process, which destroys or eliminates all forms of life, microorganisms in particular. Conventional sterilization techniques, such as those which utilize autoclaves, ovens, and chemicals like ethylene oxide (EtO), tend to rely on irreversible metabolic inactivation or on the degradation of vital structural components of the microorganism for the efficacy (Moisan *et al.*, 2002). Substantial research, however, has recently focused on plasma-badessterilization technique. Plasma is a partially ionized, low-pressure gas, which is composed of ions, electrons, and ultraviolet photons, as well as reactive neutral species (radicals and excited atoms and molecules) with energy sufficient to break covalent bonds and initiate a variety of chemical reactions (Moisan *et al.*, 2002). One of the primary advantages presented by the plasma method is the possibility, under appropriate conditions, of achieving such a process at relatively low temperatures ($\leq$ 50°C), thereby allowing for the preservation of the integrity of polymer-based instruments, which cannot be subjected to autoclaves and ovens (Adler *et al.*, 1998; Moisan *et al.*, 2001). In addition to the obivious advantage of not damaging the articles to be sterilized, cold plasmas have proven quite effective, owing to the synergistic effects of free radicals and UV photons, which interact with microorganisms on the atomic and molecular levels. It is via the combined effects of plasma processes including chemical degradation, UV photodesorption, and ion bombardment, which effects the destruction of both microorganisms and organic matter (Takeshita *et al.*, 2003; Park *et al.*, 2004). Plasma sterilization operates differently due to its specific active agents, namely ultraviolet photons and radicals (atoms or assembly of atoms with unpaired electrons, therefore chemically reactive, e.g., O and OH, respectively). During plasma treatment, microorganisms are eliminated as the consequence of direct contact with ions of high kinetic energy and electrons, in addition to UV rays. Although UV light is the primary sterilization factor in plasma sterilization, its effects are somewhat 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.

Whereas the biological effects of reactive oxygen species (Davies, 1987; Peskin, 1997) and UV radiation (Hieda *et al.*, 1994) have been determined in some detail, the biomolecular effects of low-temperature plasmas have yet to be elucidated. The primary ob-

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jective of this study was to characterize the biomolecular effects of an atmospheric-pressure cold plasma (APCP) system which employs elium/oxygen (He/O_2). The effects of cold plasma on bacteriophage DNA, nd model proteins were assessed in this study, via both crobiological, nd biochemical techniques. To accomplish this, helium gas was selected due to its ack of chemical activity and its lack of specific effects on microorganisms, espite ntense ion and electron bombardment; oxygen gas was used to augment he sterilizing effect.

A plasma sterilization system was constructed as described previously (Lee *et al.*, 2006). In brief, this system employs a 10-20 kHz and 4-20 kV generator to excite helium (He) and oxygen (O_2) gas, which is injected from a gas container into a plasma generator (400 × 500 × 3 mm) at a constant flow rate. The gas was excited to a plasma state at a frequency of 10 kHz and a power energy input of 6 kV, by passing it through a pair of He/O_2 plasma generators, with a filter or carrier positioned between the two plasma generators. The two plasma generators were positioned 6 mm apart. In this system, the Optical emission spectrum (OES) of the He/O_2 plasma generated peaks at 706 nm for the helium atom and at 777 nm for the oxygen radical, whereas no peaks were generated at 220-280 nm, in the normal spectrum of UV irradiation. The results of this study demonstrated that APCP using He/O_2 generates a low level of UV, while generating reactive oxygen radicals that probably function as the principal factor in sterilization; these reactive oxygen radicals also have the advantage of access to the interiors of complicated structures, such as those of tubes or of artificial organs. Moreover, this new system did not generate excessive heat when utilized for up to 1 h of treatment.

In order to determine the impact of low-temperature plasma on biomolecules, viral DNA, BSA, and *E. coli* cells were exposed to He/O_2 plasmas for various duration, and the effects were assessed via electrophoresis or biochemical assays. The results would be expected to contribute to our understanding of the mechanisms inherent to low-temperature plasma-induced sterilization and, the degradation of biological matter. Viral DNA solutions containing 10 g of M13 mp18 double-stranded DNA were spotted onto TLC plastic sheets (1 × 1cm, 25 TLC plastic sheets Silica gel 60 F254, MERCK) and dried at room temperature. The inoculated TLC sheets were exposed to He/O_2 plasma (200 V, O_2 50 sccm and He 5 slpm) for differing durations. After each exposure, the sheet was placed into a microfuge tube, and dissolved in 250 μl of TE buffer (pH 8.0) and gently vortexed in order to remove the samples from the sheets. In the control experiments, 80-87% of intact DNA was recovered from the TLC sheets. To precipitate the silica, the DNA samples were centrifuged for 2 min at 2,000 × g. The DNAs in the supernatant were analyzed in parellel via 0.8% agarose gel electrophoresis. Samples left unexposed to plasma were employed as controls and were maintained at room temperature while the samples were treated. In all cases, the biological molecules were solubilized from the exposed silica surfaces using buffer.

The extents of biochemical damage induced by plasma exposure for increasing time periods were determined by gel electrophoresis, and these findings are provided in Fig. 1. The amount of supercoiled DNA decreased by 40-50% after 10 sec of exposure. The new band, a lower band seen in lanes 2 and 3, accumulated after the 10 sec exposure. This would indicate that nicked or linearized DNA had been generated, owing most probably to a single- or double- stranded break. Exposure for longer than 20 sec resulted in the complete degradation of the intact DNA (circular supercoiled DNA), and as a result of multiple fragmentation, smeared bands were observed in the lower regions of the gel, in lanes 4, 5, and 6. This shows that a single-stranded break had occurred in the supercoiled DNA, and that He/O_2 plasma effected a complete degradation of the DNA.

The damaging effects on polypeptides induced by exposure to plasma were also assessed using bovine serum albumin (BSA), which is the model structural protein, as well as alkaline phosphatase, a model enzyme protein. In order to achieve this, BSA (10 μg) dissoved in 20 mM Tris-HCl (pH 7.0) was loaded onto a TLC plastic sheet (0.5 × 1 cm) and the dried film

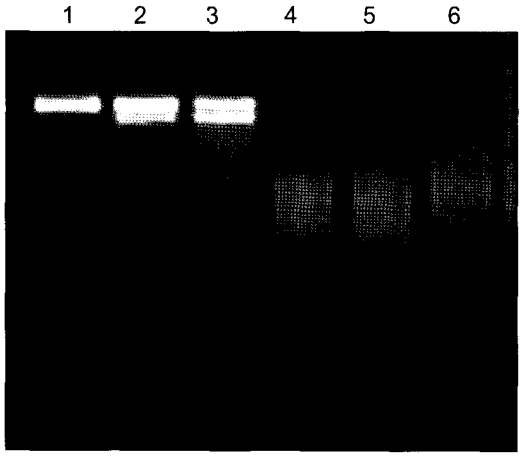

Fig. 1. Decomposition of M13mp18 DNA at various exposure times with atmospheric pressure cold plasma, using He/O_2. The circular double-stranded DNA was analyzed by 0.8% agarose gel electrophoresis. Lanes: 1, unexposed DNA ; 2, exposed 5 sec; 3, exposed 10 sec; 4, exposed 20 sec; 5, exposed 30 sec; 6, exposed 40 sec.

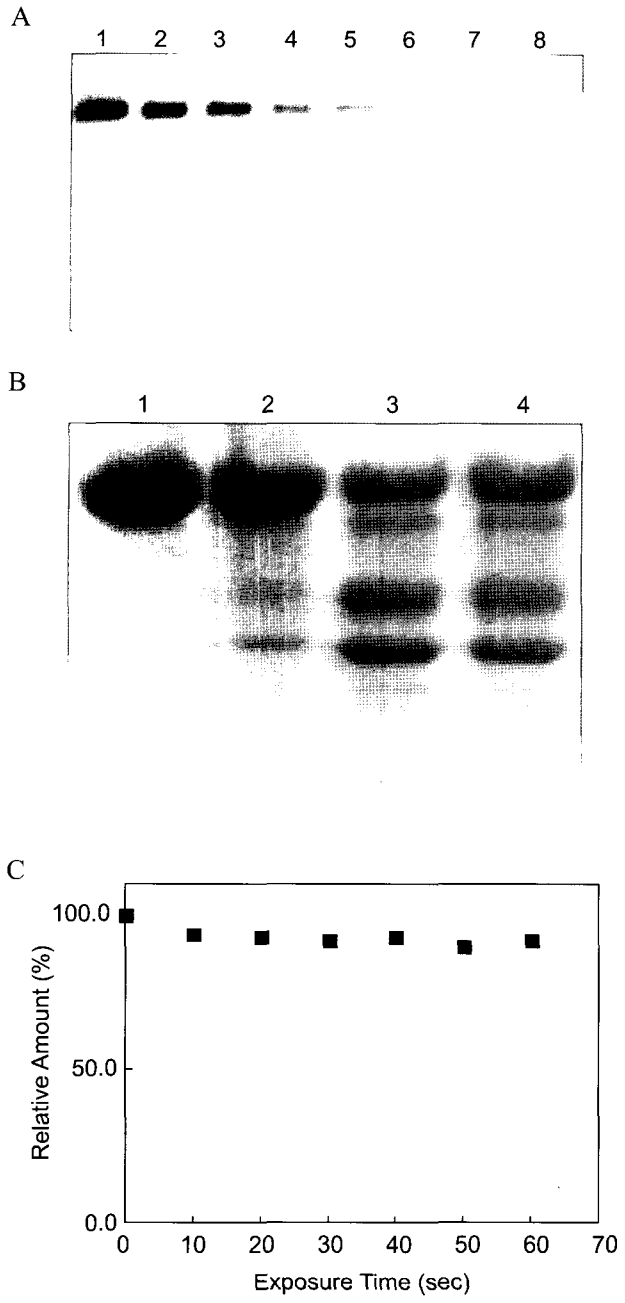

Fig. 2. (A) The effect of direct plasma exposure on bovine serum albumin (BSA). After plasma treatment for the indicated times, the protein samples were analyzed via 10% SDS-PAGE. Lanes: 1, protein samples without spotting onto TLC sheet; 2, unexposed and spotted protein sample-; 3, protein samples exposed 10 sec; 4, protein samples exposed 20 sec; 5, protein samples exposed 30 sec; 6, protein samples exposed 40 sec; 7, protein samples exposed 50 sec; 8, protein samples exposed 60 sec. (B) The effect of direct plasma exposure for a short time period on BSA. Lanes: 1, unexposed protein sample; 2, protein sample exposed 1 sec; 3, protein sample exposed 2 sec; 3, protein samples exposed 3 sec. (C) Measurement of the peptidic amino acids of the protein samples treated with plasma for the indicated time, by Bradford assay. The measurable amount in each protein sample was normalized to that of the unexposed cells.

was exposed to plasma for the indicated times. After each of the exposures, the sheets were placed into microfuge tubes and were dissolved in 300 μl of 20 mM Tris-HCl (pH 7.0), then vortexed gently in order to remove the samples from the sheet. In order to precipitate the insoluble gel of the TLC sheet, the samples were centrifuged for 2 min at 10,000 × g. The proteins in the supernatants were evaluated for changes in the integrity of proteins in parallel by SDS-PAGE, and the amount of peptidic amino acids in the protein samples was determined by Bradford assays. Samples left unexposed to plasma were employed as controls and were maintained at room temperature, whereas the samples were treated as described above in the DNA experiments. The band intensities for initial size and intact BSA diminished progressively with time (Fig. 2A). Increasing exposure times induced a nonlinear reduction in the quantity of intact BSA, until each protein band fell below the level of detection. Exposure of longer than 40 sec to BSA on silica gel resulted in the complete degradation of the intact BSA. The polypeptide species corresponding to the shorter BSA fragments were not observed in the experiments (lane 3-5) for comparably longer plasma exposure durations. However, the measureable amount of peptidic amino acids, as determined by Bradford assay, was nearly the same in all samples (Fig. 2C). This would indicate that the total quantity of the peptidic amino acids remained constant in samples that were exposed to He/O_2 plasma for durations of longer than 10 sec. However, the quantities of intact BSA decreased with increasing plasma exposure durations. The damages in intact BSA effected by exposure to plasma for short time intervals are shown in Fig. 2B. The shorter BSA fragments were generated within 3 sec of plasma exposure. The observed loss of the intact form of BSA in the sample protein and kinetic function were both attributed to the fragmentation and removal of the protein, as a result of the He/O_2 plasma chemistry. Considering the results of Fig. 2A and B, it might be concluded that the reactive oxygen species (ROS) generated in the plasma reaction targeted one protein molecule, and a subsequent chemical reaction occurred rapidly in that protein molecule rather than another new molecule of protein. Thus, multiple fragmentation occurred in the protein molecule.

The effects of plasma not only on the physical changes in proteins, but also on the enzymatic activities of proteins, were also determined. Alkaline phosphatase solution (2744 units/ml) was added to TLC sheets in 10 μl aliquots, dried at room temperature, and exposed to He/O_2 plasma. After exposure, each of the samples was dissolved in 300 μl of 20 mM Tris-HCl (pH 8.0). Aliquots in the supernatant fraction after centrifugation (2,000 × g, at 4°C for 2 min)

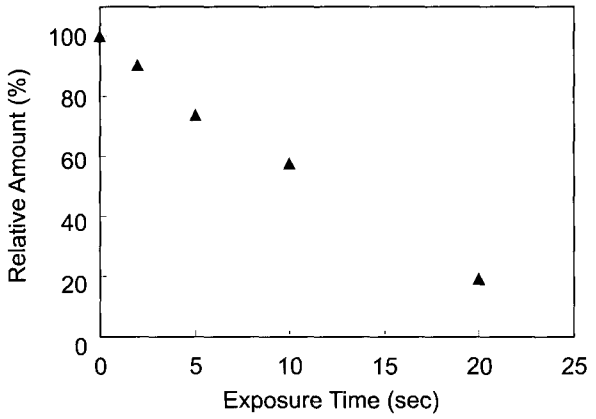

Fig 3. The effects of direct plasma exposure for the indicated time on the enzyme activity of alkaline phosphatase. Measurable activity in each enzyme sample was normalized to that of the unexposed cells.

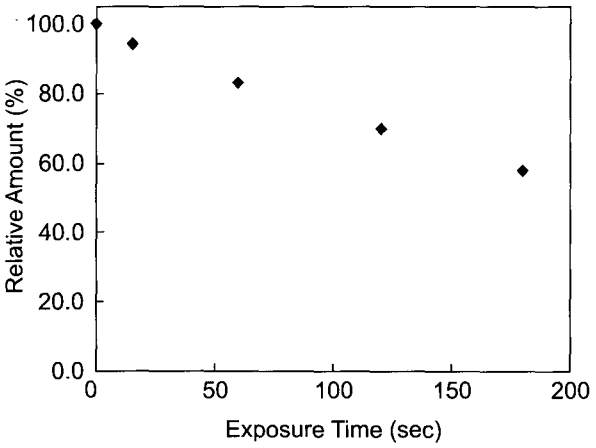

Fig. 4. The effect of plasma exposure on catalase activity in the cell. After bacterial cells were exposed to plasma for the indicated times, the activities of intracellular catalase were determined. The measurable activity in each enzyme sample was normalized to that of the unexposed cells.

were placed into fresh microtubes and employed as enzyme samples. Alkaline phosphatase activity was determined using 10 mM p-nitrophenyl phosphate and 20 mM Tris-HCl (pH 8.0). The enzymatic reactions were initiated by the addition of 30 μl of the enzyme sample, and the changes in absorbance were monitored at 403 nm. Enzymatic rates were determined by calculating the initial slopes in the progression curves of the phosphatase reaction for 30 min. Enzyme activity was reduced in the enzymes in a time-dependent manner (Fig. 3). The 80% of enzyme activity was lost in the sample exposed to plasma for 20 sec. Exposure to plasma for 30 sec resulted in the complete abrogation of enzyme activity. This shows that plasma exposure caused the rapid destruction of the protein structure of the enzyme, and ultimately resulted in the loss of enzyme activity.

The effects of plasma exposure on the biological macromolecules within the cell were also assessed. *E. coli* JM109 was grown to mid-log phase in LB medium at 37°C. The cells were harvested and re-suspended in a sterilized saline solution (10^7 cells/ml), and 50 μl were transferred to a silica gel-coated TLC sheet. The dried TLC sheets (1 × 2 cm) inoculated with *E. coli* were exposed to He/O_2 plasma for differing time periods. Samples left unexposed to plasma were employed as controls. Another control experiment indicated an 88 % recovery from the TLC sheets, with negligible effects occurring as the result of drying, as well as the heat generated within the cold plasma reactor. After each exposure, the sheet was placed into a microfuge tube containing 1 ml of 0.05 M potassium phosphate buffer (pH 7.0), and vortexed gently in order to remove the cells from the sheets. After 2 min of centrifugation at 800 × g at 4°C to precipitate the silica gel, the supernatant (1 ml) was transferred to a new tube, and lysozyme (0.1 mg/ml) was added and incubated for additional 10 min at 37°C. After the bacterial solutions were subjected to 1 min of sonication at 4°C to lyse the bacterial cells completely, the crude extracts were centrifuged for 10 min at 12,000 × g at 4°C. The recovered supernatans were then used for the source solution for the target enzyme protein (catalase). Catalase activity was determined in a reaction mixture (1 ml) containing 634 μl of 0.05 M potassium phosphate buffer (pH 7.0), and 333 μl of 0.059 M hydrogen peroxide in 0.05 M potassium phosphate (pH 7.0), as well as the enzyme (prepared as above). The enzymatic reactions were initiated by the addition of 33 μl of the enzyme sample, and changes in absorbance were monitored at 240 nm. The enzymatic rates were determined by calculating the initial slopes in the progressine curves of the phosphatase reaction for 10 min. Catalase activity, which was the target for our examination of the effects of plasma exposure on intracelluar enzyme protein, was abrogated in an exposure time-dependent manner. The total intracellular activity was reduced by 40% in cells exposed for 180 sec (Fig. 4). This demonstrated that the cell membrane could not protect against plasma penetration. Structural deformation in the cell wall and membrane occurred via the activity of O_2 plasma (Mogul *et al.*, 2003). This phenomenon was also observed in this experiment, and was thus attributed to the vulnerability of cell membranes to plasma. Thus, the impact of He/O_2 plasma in intracellular macromolecules occurred via the penetration of the cell membrane. In our comparison of enzyme proteins that were exposed directly to plasma (data in Fig. 3),

longer times were required to effect reductioons in enzyme activity of intracellular proteins. The observed differences in inactivation rates between proteins exposed directly to plasma and proteins within cells exposed to plasma were most likely attributable to shielding afforded by the structural components of the cells. This shielding effect may be the reason that longer exposure times are necessary to achieve the reduction of enzyme activity within cells to a similar extent.

To evaluate the physicochemical changes occurring in the cell, changes in the total amount of intracellular ROS were assessed after exposure to He/O_2 plasma. *E. coli* cells resuspended in saline were dried onto silica gel TLC plates (1 × 2 cm) and were exposed to He/O_2 plasma for 10, 30, and 60 sec. After each exposure, the sheet was placed into a microfuge tube containing 1 ml of 0.1 mM EDTA (pH 8.0) and vortexed gently to remove the cells from the sheets. After 2 min of centrifugation at 800 × g and 4°C to precipitate the silica gel, the supernatant (1 ml) was transferred to a new tube. The *E. coli* cells were harvested by 5 min of centrifugation at 10,000 × g, then resuspended with 0.2 ml of saline solution. After the addition of 0.2 ml of 2 mM 2', 7'-dichlorofluorescin diacetate (DCFDA), the cells were incubated for 30 min at room temperature. The fluorescein-treated cells were then collected by 10 min of centrifugation at 10,000 × g, and resuspended with 0.2 ml of saline solution. In oredr to remove the unpenerated 2', 7'-dichlorofluorescin diacetate, which was outside of the cell, the above centrifugation steps were repeated twice, and the precipitated cells were dissoved in 1 ml of cell lysis buffer (0.5 M NaCl, 0.2 M Tris-HCl (pH 7.0), 0.01 M EDTA, 1% SDS). In order to lyse the cells, glass beads (106 microns and finer, Sigma) were added to the cell suspension, and applied to a bead beater (2,500 rpm, 200 sec). The supernatant was recovered from 5 min of entrifugation at 12,000 × g. The relative amount of intracellular ROS that reacted with 2',7'-dichlorofluorescin diacetate was quantitated using a spectrofluorometer (SFM25, Kontron Instruments) at an excitation wavelength of 485 nm and an emission wavelength of 530 nm. DCFDA has a permeability to the membrane (Jakubowski and Bartosz, 2000), and is ablr to access the lipid membrane via diffusion. The DCFDA in the cell would undergo deacetylation and be transformed into a nonpermeable DCF, which would then be captured inside of the cell. Nonfluorescent DCF would react with intracellular ROS and be oxidized into a strongly fluorescent DCF. The cellular quantity of ROS in the cell exposed to He/O_2 plasma increased with increasing plasma exposure times (Fig. 5). The levels of ROS in the cell were 3-fold higher after 60 sec of exposure than in the unexposed cells.

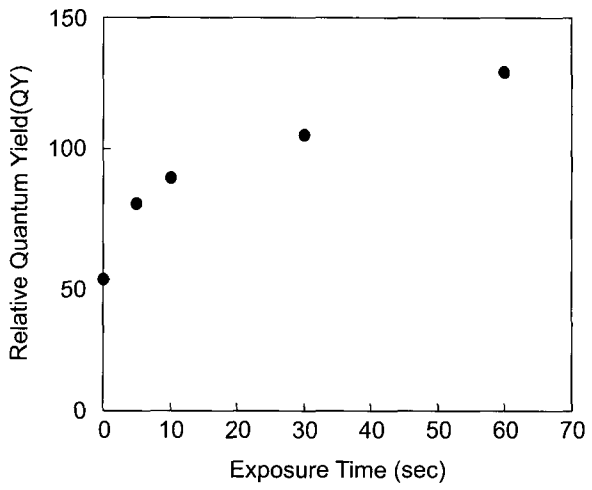

Fig. 5. The effect of plasma exposure on the quantity of intracellular ROS. After the bacterial cells were exposed to plasma for the indicated times, the levels of intracellular ROS were determined using DCFDA with a spectrofluorometer. The relative quantum yield values are shown.

This indicated that plasma exposure induced an increase level of ROS in the cells, and that the ROS was generated via the activity of plasma through the membrane, or penetrating directly into the cell. These increased ROS levels might be directly involved in the process of sterilization.

Several important mechanisms occur during the plasma sterilization process (Bol'shakov *et al.*, 2004). These include 1) chemical reactions, e.g. with atomic oxygen, 2) UV-induced damage or photodesorption, and 3) ion sputtering. Most probably, these processes function in synergistic manner, as occurs typically in semiconductor etchers, in which the removal of material by combined chemical and ionic species is significantly more rapid than removal by either species alone (Lieberman and Lichtenberg, 1994). A similar synergistic effect has been proposed in the context of UV-assisted chemical etching techniques (Leerungnawarat *et al.*, 2000). Several studies (Moisan *et al.*, 2001; Moisan *et al.*, 2002) have reported that UV radiation performs a primary function in biological sterilization, particularly under low pressures. These studies characterized the effect of UV in terms of microbial mortality, rather than in terms of biomaterial degradation and removal efficiency. According to the results obtained in tbis study, it can be concluded that, under our experimental conditions, the time required to achieve the complete inactivation of biological macromolecules is related more closely with ROS density than with UV radiation intensity. In a previous study (Lee *et al.*, 2006), it was demonstrated that APCP using He/O_2 generates low UV levels while generating reactive oxygen radicals that

probably function as the main factor in sterilization; these reactive oxygen radicals carry the advantage of being able to access the interiors of complicated structures. The adsorption of reactive species from the plasma on the microorganism with which they subsequently undergo chemical reactions This finding supported the aforementioned hypothesis, which proposed that He/O_2 plasma exerts a sterilizing effect via the activity of oxygen radicals, which are able to access and directly attack the cell wall and intracellular macromolecules. Further research will be necessary in order to determine the manner in which oxygen radicals contribute to the inactivation process, resulting in the death of microorganisms.

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