Sterilizing Ability of High-Voltage Pulsed Discharge Plasma with Cavitation for Microorganisms Including Radio-Resistant Bacterium in Water

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Received 13 May, 2021/Accepted 1 September, 2021

There are various purification methods have been developed and applied to industrial wastewater with contaminated microorganisms. We previously reported that high-voltage pulsed discharge plasma with cavitation effectively kills Escherichia coli cells. We attempted to expand the application of this disinfection method by using microorganisms such as Bacillus subtilis, Deinococcus radiodurans, and Schizosaccharomyces pombe. These microbial cells were treated with the discharge plasma, and the cell viability, DNA damage, and morphological changes were analyzed to evaluate the bactericidal effect. Interestingly, D. radiodurans, a radio-resistant bacterium showed relatively high sensitivity to the discharge plasma. On the other hand, B. subtilis and S. pombe showed the resistance, showing both sporogenesis. The amount of DNA damage in the treated cells corresponded to the cell viability, but most of the treated cells did not show any morphological changes.

Key words : Plasma / Pulsed discharge / Sterilization.

Wastewater becomes a source of environmental pollution and causes adverse health effects including direct and infectious illness. Therefore, wastewater control is one of the required procedures for our social and industrial activities since mankind has been releasing it with our various activities from ancient times. Against the wastewater from manufacturing, agriculture, mining, transportation, and food processing, we have developed and constructed the various cleaning systems including physical, chemical, and biological procedures. Among these cleaning procedures, plasma technology is one of useful wastewater control (Bruggeman et al., 2016). We previously indicated that pulsed discharge plasma is a good candidate for water purification (Kudo et al., 2015a; 2015b). In those studies, we constructed a model circular apparatus with generating pulsed discharge plasma, and treated Escherichia coli cells for evaluation of the sanitization ability. The treated E. coli cells were killed along the input energy, and the oxidative DNA lesions were produced in their chromosome. The DNA damage includes oxidative base lesions detected by specific DNA glycosylases for oxidative pyrimidines and purines. We also investigated that the generation of reactive oxygen species (ROS) by the plasma. Then, we conclude that the plasma effectively kills the E. coli cells by producing ROS via the DNA damaging. Our previous study shows the possibility of the plasma technique for sterilization.

In this study, we examined the effect of high-voltage pulsed discharge plasma with cavitation on the bactericidal ability of wide range of microorganisms for wastewater purification. We here treated Bacillus subtilis, Deinococcus radiodurans, and Schizosaccharomyces pombe by this plasma apparatus. B. subtilis is one of the most common bacteria with gram-positive. D.
radiodurans is a radioresistant bacterium found from rotten canned meat despite radiation sterilization (Anderson et al., 1956). The sterilization of radioresistant bacteria including D. radiodurans is quite difficult because we cannot use ionizing radiation for this purpose. S. pombe is the only eukaryote sample in this study. This is the model for sterilization for eukaryotic contamination because yeast containing this species is also a major source of biological contamination. Additionally, B. subtilis and S. pombe can produce spores for their dormancy against environmental deterioration. The spore form usually shows a higher resistance ability for various sterilizing factors than the vegetative form of respective species with sporulation.

The microbial species used in this experiment are as follows. E. coli K12 was our laboratory stock (Kudo et al., 2015b). B. subtilis subsp. subtilis 1465 was obtained from Japan Collection Microorganisms. D. radiodurans KR1 is an original strain (Anderson et al., 1956), as a gift from Dr. Takeshi Saito (Kyoto University). S. pombe HT02 was established, and given to us by Dr. Shogo Ikeda (Okayama University of Science) (Kanamitsu et al., 2007). The medium for B. subtilis was #22 medium containing 10 g/L of tryptone, 10 g/L of beef extract (Difco, United States), and 5 g/L of NaCl, and adjusted the pH7.0. D. radiodurans cells were cultivated in TSA medium (Eiken Chemical, Japan). The media for E. coli (Kudo et al., 2015b) and S. pombe (Kanamitsu et al., 2007) were described previously. The cells of tested microorganisms were cultivated with shaking aeration (200 rpm) until the OD_{600} reached 0.3. E. coli cells were grown at 37°C, and the other microorganisms were at 30°C. The cultivated cells were harvested, then washed twice in phosphate buffered saline (PBS) (Nacalai Tesque, Japan), and resuspended in the same solution. The cells were suspended in 2 L of distilled water to 10^6 colony-forming units per milliliter (CFU/mL). The cell suspension was first circulated for 2 min at a flow rate of 4.6 mL/min to ensure the cells were well mixed before initiating the plasma treatment. Then, the suspension was circulated at a flow rate of 20 L/min and was treated by the plasma up to 90 seconds. The treated cells were sampled at 0, 30, 60, and 90 seconds for the analyses for cell viability, DNA damage, and morphology mentioned below. The apparatus generating pulsed discharge plasma with cavitation consists of a plasma reactor, a power supply for discharge generation, and water-circulation equipment containing a 2 L-water vessel and water pump (Fig. 1). An alternative current voltage from the power supply is 10 kHz frequency. The typical waveforms are shown in Fig. 2. The plasma reactor equipped with two electrodes for discharge and a nozzle of 3 mm diameter to produce water cavitation.

In the reactor, the high-voltage electrode was set at 13 mm from the nozzle, and the grounded electrode is 4 mm away from another one. The calculated cavitation number (δ) was set as the reference (Gogate and Pandit, 2000). The estimated electric strengths at the center between two electrodes and in the vicinity were 2.5 and 6 kV/cm, respectively. The more details of this plasma apparatus and experimental conditions were described previously (Kudo et al., 2015a; 2015b).

For cell viability analysis, the plasma-treated cells were appropriately diluted with PBS. Then, the cell suspensions were grown on the respective agar plate media, and the colonies were scored for surviving fractions. For DNA damage analysis, the chromosomal DNA in the plasma-treated cells was analyzed by static field gel electrophoresis (SFGE), as described previously (Kudo et al., 2015a). For only S. pombe cells, a step of overnight pretreatment with 2 μg/mL zymolyase in PBS was added at 25°C. For cell morphological investigation, the plasma-treated cells were fixed in 2.25% (v/v) glutaraldehyde overnight, and then dehydrated by sequential ethanol soaking from 50% to 99% (v/v) of ethanol and tert-butanol. The dried cells were coated with gold by spattering apparatus (IB-3, Eiko Engineering, Japan), and observed by the scanning electron microscope (JSM-6510, JEOL, Japan).

![FIG. 1. The apparatus for generating pulsed discharge with cavitation. This apparatus consists of water vessel for 2 L of treated water (A), water pump (B), plasma reactor equipping a grounded and high-voltage electrode with nozzle making cavitation (C), and power supply (D). Arrows and numbers indicate the flow path, its direction, and the order of flow. The details for the apparatus are in the text.](image)
the decimal reduction time (D value) which is the time required to kill one log (90%) by the plasma are 28.78, 42.64, 227.6, and 262.9 seconds for D. radiodurans, E. coli, B. subtilis, and S. pombe, respectively. Thus, one is the plasma-sensitive group including E. coli and D. radiodurans, and another group is resistant to plasma including B. subtilis and S. pombe. It is interesting that the radioresistant bacterium was almost as susceptible as E. coli, or even more so. Because radioresistant bacteria are usually resistant to a variety of damaging factors. This result suggests that the plasma technique is effective as a sterilization method for radioresistant bacteria. On the other hand, it is also interesting to note that both B. subtilis and the fission yeast were resistant to the plasma. Both resistant species show sporulation, although these are classified into prokaryotic and eukaryotic species, respectively.

Next, we investigated the mechanism of plasma-induced cell death by observing the chromosomal DNA damage by SFGE. Both susceptible E. coli and the radioresistant bacterial cells showed the generation of double strand breaks (DSBs) of their chromosomal DNA with plasma treatment time (Figs. 4A, B). SFGE technique shows chromosomal DNA fragmentation as migrated bands on the electrophoresis gel indicating DSBs generation (Terato et al., 2008). Comparing the results of E. coli and D. radiodurans, the amount of DNA damage seems to be higher in the latter. This is consistent with the results for their susceptibility (Fig. 3). These results indicate that this DNA damage was caused by the plasma. On the other hand, B. subtilis and the fission yeast, which showed resistance to plasma, did not show DSB generation (data not shown). This suggests that the bactericidal mechanism by plasma is derived from DNA damage, which is consistent with our previous findings (Kudo et al., 2015a; 2015b). Thus, the chromosomal DNA lesions can cause cyto-
plasma may directly damage intracellular DNA like ionizing radiation.

Regarding the sterilization mechanism of plasma, we reported in a previous study that plasma-generated ROS plays an important role (Kudo et al., 2015b). The ROS are of plasma origin from our apparatus utilizing cavitation to generate plasma easily because we did not observe ROS generation by cavitation alone with energization on the apparatus (Kudo et al., 2015b). ROS are also important agents in the biological effects of ionizing radiation (von Sonntag, 1987). In the case of radiation biological effects, the starting material for ROS is water, which is rich in living organisms and generates many types of ROS, including hydroxyl radical (Jiang et al., 2014; Gupta and Bluhm, 2007). We observed in the previous study that plasma can produce ROS (Kudo et al., 2015b). The energy dosage required to produce 30 nm/mL of hydroxyl radicals is 1,250 mJ/mL, and the energy to produce the same amount of hydrogen peroxide is 100 mJ/mL. Like water radiolysis, it can be assumed that hydroxyl radical is also the primary product in water splitting by the plasma, and similarly hydrogen peroxide is thought to be the secondary product produced by the recombination of the primary products such as hydroxyl radical (von Sonntag, 1987). Therefore, the difference in the energy requirements mentioned above means that more than 90% of the hydroxyl radicals are quickly converted to hydrogen peroxides. ROS generation in plasma is thought to be a region of 1 mm in diameter sandwiched between two electrodes, and the hydroxyl radicals densely generated in this narrow region are expected to easily recombine to produce a large amount of hydrogen peroxide.

Toxicity with the plasma, but it is still unclear whether there are other pathways causing cytotoxicity. The observation by scanning electron microscopy showed no deformed cell images in the resistant B. subtilis and the fission yeast (data not shown). The sensitive E. coli and D. radiodurans also showed that most of the cells remained intact (Fig. 5). These results suggest that
peroxide. Therefore, the apparent yields of hydroxyl radical and hydrogen peroxide were small and large, respectively. In fact, the importance of hydrogen peroxide in discharge plasma in water has been recognized (reviewed on Locke and Shih, 2011). In our case, the highly reactive hydroxyl radicals are small in quantity and outside the cell, so how can the plasma be cytotoxic? Extracellular hydroxyl radical cannot pass through the cell membrane and will only damage the outside of the cell membrane. However, we observed that while the treated cells were plasma sensitive, they did not exhibit any morphological changes (Fig. 5). Hydrogen peroxide is electrically neutral and can pass through the cell membrane. The most of hydrogen peroxide generated outside the cell can enter the cell and is involved in DNA damaging (Chance et al., 1979). Because of its low reactivity, hydrogen peroxide is unlikely to damage directly (Takemoto et al., 1998). There are two possible pathways by which low-reactivity hydrogen peroxide may be involved in DNA damage. The first pathway is the one involving ultraviolet (UV) light. Hydrogen peroxide reacts with UV light to produce hydroxyl radicals (Sato, 2009). The plasma emits UV light, and the UV light generated can activate hydrogen peroxide, resulting in reproduction of hydroxyl radicals. It was reported that UV generated by underwater discharge plasmas decomposes hydrogen peroxide and contributes to 30% of the bactericidal effect (Lukes et al., 2008). It suggests that the cooperation between UV and hydrogen peroxide plays an important role in this study. Another pathway is the interaction of hydrogen peroxide and intracellular metal ions. Hydrogen peroxide reacts with metal ions to produce hydroxyl radicals as the Fenton reaction (Walling, 1975). The Fenton reaction was first discovered with iron ions, but it was later discovered that similar reactions can occur with a variety of transition metals (reviewed on Kawanishi et al., 2002). Since there are a variety of metal ions in the cell, these metal ions may react with intracellular hydrogen peroxide to generate highly active hydroxyl radicals that attack intracellular biomolecules. Generally, it is thought that plasma produces hydroxyl radicals outside the cell, and the hydrogen peroxide produced by the recombination passes through the cell membrane and enters the cell, where it reacts with UV light and metal ions to regenerate hydroxyl radicals and can cause DNA damage. Plasma can generally produce a lot of kinds of cytotoxic factors, including ozone, but we do not discuss ozone toxicity because we have not observed its production in this system (Kudo et al., 2015b).

In this study, plasma was shown to be extremely effective against the radioresistant *D. radiodurans* (Figs. 3, 4B). *D. radiodurans* shows 50-100 times higher resistant than *E. coli* to gamma-rays (Moseley and Mattingly, 1971). The radioresistant bacterium shows universally highly resistant to non-radiation cytotoxic agents, (reviewed on Battista, 1997), and it also shows resistant to hydrogen peroxide with its catalase activity (Wang and Schellhorn, 1995). The catalase activity is inducible, and its induction requires a time unit of hours. In general, chemical susceptibility of microorganisms is evaluated by immersing them in the chemical agent for a certain period of time, but in this plasma treatment, inductive resistance may not be expressed because of the extremely short exposure to the damaging factor. We need a further study to determine such as how hydrogen peroxide reacts with intracellular metals to generate hydroxyl radicals that damage DNA. We will continue to study the plasma susceptibility of the radioresistant bacterium, not only because of the possibility of inadequate response to hydrogen peroxide, but also because there are still many questions that need to be answered, such as what kind of DNA damage is involved.

On the other hand, this plasma method was not effective against *B. subtilis* and *S. pombe*, both are spore-forming organisms. These two species seem to have nothing in common, as the former is a prokaryote, and the latter is a eukaryote. In spore-forming species, it is generally known that the spore stage is more resistant to various damaging factors than the trophoblast stage. The $D_{37}$ of *B. subtilis* trophoblast to X-rays is 50 Gy, while that of spores is reported to be 450 Gy (Zamenhof et al., 1965). The susceptibility of *B. subtilis* trophoblast cells to radiation is almost equal to that of *E. coli*, but the sporulation enhances the resistance as tenfold. The $D_{37}$ of the fission yeast to gamma-rays is 800 Gy (Nasim and Smith, 1975). Given that radiobiological effects are mediated by ROS, it is understandable that *B. subtilis* and the fission yeast are plasma-resistant as well as radioresistant. *D. radiodurans* does not show sporulation. Thus, sporation formation seems to be one of the resistant factors. Whether the plasma-resistance of their spores’ morphology is due to their internal structure or to the inhibition of hydrogen peroxide permeation by the spore cell wall requires further investigation.

In conclusion, this study showed a high effect of plasma on the radioresistant bacterium and a low effect on *B. subtilis* and the fission yeast. Plasma is expected to be useful as a new means of sterilization against radiation-resistant bacteria and needs more ingenuity to apply to sporulating species.

**ACKNOWLEDGMENTS**

We appreciate Prof. Shogo Ikeda (Okayama University
of Science) and Dr. Takeshi Salto (Kyoto University) for gifts of microorganism strains. We also thank Ms. Kanee Mori (Saga University) for her technical supporting. This study was supported by The Yakumo Foundation for Environmental Science (HT), and Kurita Water and Environment Foundation (YT). This study was partly supported by JSPS KAKENHI Grant Number 20K04446 (SI).

CONFLICT OF INTEREST

The authors declare no conflict of interest.

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