Peroxynitric acid ($HOONO_2$) chemistry inside plasma-treated water (PTW) for effective and safety disinfection

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Abstract: Plasma-treated water (PTW) has been attracting attention in the field of plasma medicine. We have demonstrated that the bactericidal activity was enhanced at pH < 4.8 and the half-life was elongated by cryo-preservation. Active component in PTW has been confirmed to peroxynitric acid (PNA; $HOONO_2$), which no one has used for bacteria killing in the past. Only in thin plasma-water interface, PNA would synthesized by chemical precursors supplied by plasma. In this paper, PNA chemistry inside PTW is discussed.

Keywords: plasma medicine, disinfection, atmospheric plasma, plasma treated water.

1. The reduced-pH method for effective disinfection

For the purpose of the disinfection against human bodies for dental [1-4] and surgical applications using low-temperature atmospheric-pressure plasmas (Fig. 1), the inactivation of bacteria in water solution should be in consideration. For that purpose, the reduced-pH method was developed that strong bactericidal activity by direct plasma exposure to bacteria suspension can be achieved if the solution is sufficiently acidic [5]. Drastic enhancement of bactericidal activity is achieved by controlling the pH of the solution under 4.8, and D value (decimal reduction time) surprisingly became 1/100 when pH is changed from 6.5 to 3.8. D value (*Escherichia coli*) at acidic condition can be controlled to quite small (< 2 sec) under some condition. We call this technique as "the reduced-pH method".

It is considered that relatively strong bactericidal activity is brought by the production of hydroperoxy radical (HOO•) from the association of hydrogen ion (H^+) and superoxide anion radical (O_2^-•). The critical pH value is associated with p*K*a of the acid dissociation equilibrium between these radicals, which is known to be approximately 4.8. This well-known chemical reaction means that O_2^-• can be changed into HOO•, which have much stronger bactericidal activity, in lower pH. Penetrating HOO• brings oxidative stress in cell to be killed [6].

Fig. 1 Plasma jet exhausted to a finger without burning.

Because body fluid has neutral pH buffer capacity of pH ~7.4, bactericidal activity is limited to 1/100 if plasma disinfection is applied without pH buffer solutions. To achieve drastic plasma disinfection, this reduced-pH method is indispensable. Just before plasma treatment of infected area, acidic pH buffer solution should be applied to its surface.

Although half-life period of O_2^-• is known to be a few seconds, the reduced-pH method is also effective with PTW. Half-life period of the bactericidal activity is much longer than O_2^-•. So it is considered that unknown precursor of O_2^-• would be present in PTW.

2. The cryo-preservation method for the conservation of bactericidal activity

To understand half-life periods of PTW, PTW stored with precise temperature control were mixed to bacteria suspension. Bactericidal activity of PTW was found to decay exponentially by time. Half-lives of this activity were in accordance with Arrhenius equation in the liquid and the solid states (Fig. 2). From the experimental results of ESR (electron spin resonance) measurements of O_2^-• with spin trapping method, half-lives of obtained ESR signals were also in accordance with Arrhenius equation (Fig. 2). Both activation energies are calculated to be almost equal to ~109 kJ/mol. Half-lives at deep freezer temperature (-80 ºC) and body temperature (+37 ºC) are estimated to 7 centuries and 3.9 seconds from Arrhenius equation, respectively. This indicates that PTW can be cryo-preserved in freezer and toxicity to human body seems to be low due to fast disappearance of the bactericidal activity [7]. In addition to this temperature dependence, half-lives were found to depend also on the pH. Above absolute values of half-lives were obtained at

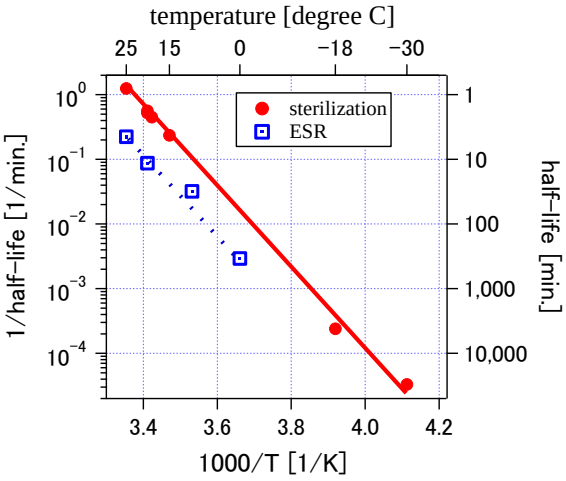

Fig. 2 Arrhenius plots for bactericidal activity and ESR measurements of PTW.

pH 4.5. From experiments at various pH condition, activation energies were same and frequency factors were different.

Although the decay mechanism of O_2^-• is known to be the disproportional reaction between O_2^-• and HOO•, our results show the simple first-order decay. Dependencies of PTW half-lives on pH and temperature dependency were quite different from these of O_2^-• (HOO•). In addition to that, no ESR signal was obtained when PTW was directly measured by ESR at liquid nitrogen temperature. So ESR signal of O_2^-• was obtained only with spin trapping chemical. These results suggest that unknown precursor of O_2^-• would be present in PTW.

3. Bactericidal activity of PTW

Considering the temperature dependency of PTW, PTW with stronger bactericidal activity would obtained by longer plasma exposure under enough low temperature, where half-life period should be much longer than plasma treatment time. PTW was continuously produced by 1 m long special plasma device equipped with cooling system. As shown in Fig. 3, *Bacillus subtilis* (spore) was inactivated in a rapid manner with high concentration PTW [7].

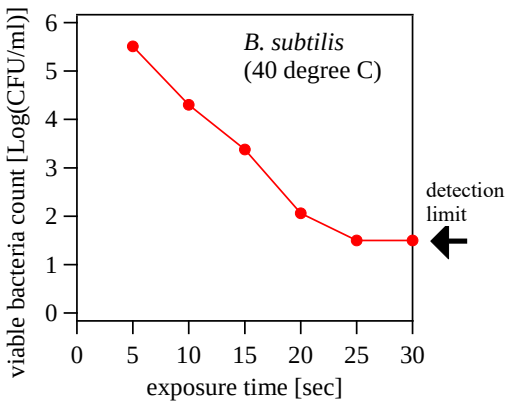

Fig. 3 Inactivation of *B. subtilis* (spore cell) with PTW at pH 3.0.

Relative bactericidal activity was estimated comparing commercially available bactericides. Serial diluted PTW or commercial bactericides were mixed with bacteria suspension. Bactericidal activity of PTW is calculated to be so high that 22 log reduction (i.e. 10^{-22}) of spore cell (*B. subtilis*) would be achieved with undiluted PTW. This corresponds to 65% hydrogen peroxide (H_2O_2), 14% sodium hypochlorite (NaClO) and 0.33% peracetic acid (CH_3COO_2H) respectively, which are deadly poison for human. Unlike such stable chemicals, bactericidal activity of PTW is inactivated quickly by body heat. Strong oxidative stress by PTW would exert only upon the surface of applied area. This property of short lifetime is expected as a novel disinfectant for human body.

For disinfection of caries cavity and root canal therapies in dentistry, PTW was applied to infected models using human extracted tooth. Only 10 sec. treatment brought reduction of cariogenic bacteria (*Streptococcus mutans*, *Enterococcus faecalis*, *Candida albicans* and *Candida glabrata*) in dentine tubules under detection limit [3, 4].

4. Identification of active component in PTW to PNA

Although the meaning of PTW is just how to generate, chemistry inside PTW should be cleared both for scientific research and commercial use. PTW was analyzed by the ion chromatograph (IC) [8]. The analysis was carried out at low temperature condition to avoid the thermal deactivation of PTW. Result of the analysis (Fig. 4) revealed that PTW contained hydrogen peroxide (H_2O_2), nitrate (NO_3^-) and nitrite (NO_2^-). In addition to these peaks, a specific peak eluted after nitrite ion was seen around at 3 min retention time. This peak was not detected in heat-treated PTW showing no bactericidal activity, suggesting that a substance contained in this peak played an important role in the bactericidal activity of PTW.

To examine the bactericidal activity of respective peaks of PTW, eluate of IC was collected respectively by 0.5 mL and bactericidal assay (*E. coli* suspension with pH 3.5 buffer) was performed with each fraction. As a result, strong bactericidal activity was observed only with

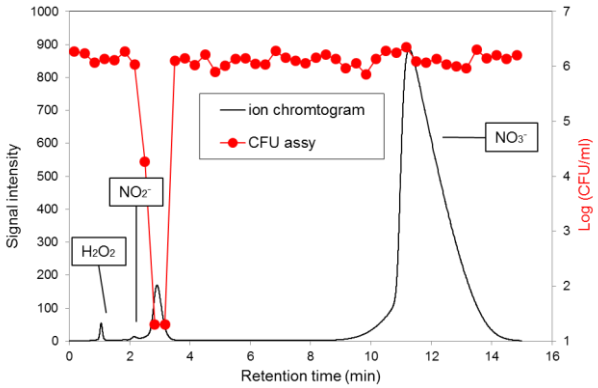

Fig. 4 Ion chromatogram of PTW and CFU assay of its each fraction.

fractions around at a PTW specific peak described above, and no bactericidal effects were observed in other peak fractions (Fig. 4). This result revealed that the bactericidal activity of PTW was due to single chemical substance, not a combined effect of plural components.

Furthermore, fractions containing bactericidal species (or its precursor) were inactivated by heating and applied to IC again. Consequently, only nitrate and nitrite were detected. This means that degradation products of the bactericidal species are nitrate and nitrite ions, strongly suggested the bactericidal species is a compound consisting of oxygen and nitrogen atoms.

Considering the activation energy for degradation of these species, we assume that peroxynitric acid (PNA; $HOONO_2$) stored in PTW induces the bactericidal effect. Our experimental results showed 109 kJ/mol and another paper reported 110 kJ/mol [9]. These are quite similar values [6].

5. Synthesis of PNA in PTW

PNA is known to be chemically synthesized by mixing HNO_2 and H_2O_2 [10]. Peroxynitric acid (HOONO) is generated from HNO_2 and H_2O_2 (equation 1) and HOONO reacts with H_2O_2 again to generate PNA (equation 2). As HNO_2 is weak acid (p*K*a 3.4), reaction rate of equation 1 depends on pH (faster at lower pH). For equation 2, nitronium ion (NO_2^+) is involved as intermediate species and NO_2^+ is generated only at extremely low pH condition. So, pH should be extremely low for chemical synthesis of PNA.

$$HNO_2 + H_2O_2 \rightarrow HOONO + H_2O \quad (1)$$

$$HOONO + H_2O_2 \rightarrow HOONO_2 + H_2O \quad (2)$$

We made PNA synthesis experiment with pH control. As shown in Fig. 5, pH < 2 at least is required to obtain PNA. But pH of usual PTW in related research is known to be about 3~4. Here, chemical species are supplied through plasma-liquid interface into water solution. Focusing on the interface, supplied chemical species through the interface diffuse deeply into water by concentration gradient. This means the concentrations of supplied primary species are high at the interface. It is not difficult to imagine that there would be extremely low pH area around at the interface and PNA could be synthesized by plasma supplied chemical species (HNO_2, HNO_3 and H_2O_2). Local distribution of pH could be seen only during plasma treatment time and no PNA would be synthesized after plasma treatment (i.e post discharge after diffusion). So this situation is not same to the simulated PTW (pH 3~4) using measured concentration parameters of averages with post-discharge PTW. In other word, our chemical synthesis experiment (Fig. 5) simulates the situation of the thin interface region. We think this is key point of PNA chemistry in PTW.

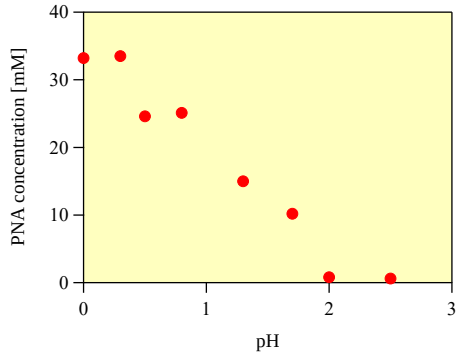

Fig. 5 Concentrations of chemically synthesized PNA at various pHs.

P. Lukes insisted the peroxynitrite acid chemistry [11]. This is quite related to our PNA chemistry. Peroxynitric acid chemistry belongs to post discharge PTW according to reaction (1). HOONO is generated slowly (for several hours) to kill bacteria. We think PNA was synthesized in usual PTW experiment including excellent experiments by P. Lukes. But, usual PTW experiments were done at room temperature, no PNA remains at such condition after a few minutes.

For understanding chemistry in PTW, pH and temperature are very important parameter respectively. During plasma treatment, PNA is synthesized rapidly around locally in thin layer of plasma-water interface, where pH is much lower than 2. Generated PNA would be stored in PTW if the temperature is enough low. For the time scale of post-discharge, HOONO is slowly generated from remaining HNO_2 and H_2O_2 in PTW. PNA chemistry is important especially in thin layer during plasma treatment and peroxynitrite chemistry is important mainly after post-discharge. Both are true and complementary, but assumed situation is just different.

6. Summary

For further understanding of plasma medicine, experimental results must be discussed based on not the parameter of plasma generation but that of key active species. In this paper, we discussed the effective plasma sterilization in liquid (direct plasma / PTW) with the reduced-pH method, based on the chemical kinetics concerning temperature. Detailed analysis of PTW concluded that key bactericidal chemical agent of cryo-preserved PTW with the reduced-pH method is peroxynitric acid (PNA). We found the use of peroxynitric acid solution as bactericidal agent at acidic pH condition for the first time in the world. PNA in PTW would be synthesized during plasma treatment around at plasma-liquid interface, where pH is quite low. If the temperature is enough low, generated PNA would be stored in PTW. These experimental results and understandings would contribute to plasma chemistry in liquid, especially to the research area of plasma medicine.

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