Peroxy nitric acid (HOONO2) is the active component in cryo-preserved plasma-treated water with the reduced-pH method for effective and safety disinfection

K. Kitano1, S. Ikawa2, Y. Nakashima2, N. Kawashita3 and A. Tani4

1 Graduate School of Engineering, Osaka University, Suita, Osaka, Japan
2 Technology Research Institute of Osaka Prefecture, Izumi, Osaka, Japan
3 Graduate School of Pharmaceutical Sciences, Osaka University, Suita, Osaka, Japan
4 Graduate School of Human Development and Environment, Kobe University, Kobe, Hyogo, Japan

Abstract: Plasma-treated water (PTW) has strong bactericidal activity under acidic condition and it can be kept by cryo-preservation. Although PTW had many chemical components, respective chemical components were isolated by ion chromatography. Based on our experimental results, we conclude that peroxynitric acid (HOONO2) is the key active component. It could be a precursor of O2–•/HOO•. Higher concentration of HOO• under acidic condition (pK\textsubscript{a} 4.8) would bring higher bactericidal activity.

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

1. Drastic plasma disinfection technique in liquid by the reduced-pH method

Considering the disinfection of human bodies for dental [1-4] and surgical applications by low-temperature atmospheric-pressure plasmas (Fig. 1), the inactivation of bacteria in liquid is essential. For that purpose, the reduced-pH method was developed that strong bactericidal activity by direct plasma exposure 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 (O2–•). The critical pH value is associated with pK\textsubscript{a} of the dissociation equilibrium between these radicals, which is known to be approximately 4.8. This well-known chemical reaction means that O2–• can be changed into HOO•, which have much stronger bactericidal activity, in lower pH [6]. Penetrating HOO• brings oxidative stress in cell to be killed.

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.

2. Conservation of bactericidal activity of plasma-treated water by cryo-preservation

In addition to direct and remote plasma exposures to bacteria suspension, the reduced-pH method can be applied to the sterilization by plasma-treated water (PTW). As shown in Fig. 2, Bacillus subtilis (spore) was inactivated in a rapid manner with high concentration PTW [7].

Bactericidal activity of PTW is known to decay exponentially. Half-lives of this activity were in accordance with Arrhenius equation in the liquid and the solid states (Fig. 3). From the experimental results of ESR (electron spin resonance) measurements [8] of O2–• with spin trapping method, half-lives of obtained ESR signals were also in accordance with Arrhenius equation (Fig. 3). Both activation energies are calculated to be almost equal to ~109 kJ/mol. Half-lives at deep freezer temperature (~80 °C) and body temperature (~37 °C) 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 [9, 10]. 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 pH 4.5. From experiments at various pH condition, activation energies were same and frequency factors were different.

Fig. 1 Plasma jet exhausted to a finger without burning.
3. Bactericidal activity of PTW

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 ideal as a 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. Purification of the active component in PTW by ion chromatograph

After high concentration PTW was prepared by 1 m long special device which can continuously irradiate atmospheric-pressure plasma to pure water flowing slowly with cooling system [11], PTW was analyzed by the ion chromatograph (IC) [12, 13]. 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 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.

From experimental results with special air tight plasma device, molecular nitrogen was found to be required both in the ambient gas and in the distilled water (dissolved gas) used to prepare the PTW [10]. We also suggest that the reactive molecule in PTW with bactericidal effects is not a free reactive oxygen species but a compound containing nitrogen atom(s). We consider that O_2• or NO_2• or NO_3^-.
HOO• is released from this compound, such as peroxynitrous acid (HOONO) or peroxynitrlic acid (PNA, HOONO2).

5. Peroxynitric acid in PTW

Many researchers are interested in this area of PTW, where the waters are treated/activated by their original devices. Many kinds of chemical species generated by plasma are supplied to water [14, 15]. Concentrations of respective chemical species contained in each PTW are different. For scientific approach, we should discuss something based on chemical species and consider that the key active component is different and dependent on each specific application.

As previously mentioned, we demonstrate that the bactericidal activity of PTW can be enhanced by the reduced-pH method and be kept by the cryo-preservation. Our approach is different from the other PTW in these respects, and this means that the key active component which we used for the disinfection possibly differs from other. For example, we think our PTW has strongest bactericidal activity than others. Respective chemical components in our PTW were clearly separated by IC and the specific peak of the key active component in PTW with our method was purified.

Considering the activation energy for degradation of these species, we assume that PNA stored in PTW induces the bactericidal effect. Our experimental results showed 109 kJ/mol and another report showed 110 kJ/mol [16]. These are quite similar values [10].

PNA is known to be obtained just by mixing chemicals, as shown in chemical reaction formulas (1, 2) [17].

\[ HNO_2 + H_2O_2 \rightarrow HOONO + H_2O \]  
(1)

\[ HOONO + H_2O_2 \rightarrow HOONO_2 + H_2O \]  
(2)

From IC analysis of chemical synthesized PNA, a same specific peak was seen at same retention time by IC and bactericidal effect was also same. At this stage, we convinced that PNA is the key active component in cryo-preserved PTW.

From the viewpoint of PTW, HNO2 and H2O2 are supplied to water from plasma chemical reactions in gas phase. On the surface of the water (i.e. interface), it seems natural that PNA would be generated from these chemical species and be stored if the water temperature is enough low.

PNA is not ROS like HOO• but RNS. Although it is known that PNA is in equilibrium with HOO•, the equilibria constant was reported with various values [18, 19]. Now we started the quantum chemical calculation on PNA decomposition to understand PNA decomposition. As a result, it was found that there are three stable structure groups next to the most stable structure (Fig. 5) with the highest existence probability. One of them has a cleavage pathway to NO2 and HOO• in the vicinity and no other pathway exists in the near energy range.

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 peroxynitrlic acid solution as bactericidal agent at acidic pH condition for the first time in the world. These experimental results and understandings would contribute to plasma chemistry in liquid, especially to the research area of plasma medicine.

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7. References