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A comparative study of the major antimicrobial agents against the yeast cells on the tissue model by helium and air surface micro-discharge plasma

Mengru Du, Hangbo Xu, Yupan Zhu, Ruonan Ma, and Zhen Jiao

AFFILIATIONS
Henan Key Laboratory of Ion-beam Bioengineering, College of Agricultural Science, Zhengzhou University, Zhengzhou 450052, China

ABSTRACT
Surface micro-discharge (SMD) plasma with a large-area and homogeneous discharge has attracted much attention in the skin disinfection due to its high antimicrobial efficiency and less side effects on tissues. Although SMD plasma sterilization is undisputedly attributed to the reactive oxygen and nitrogen species (RONS), the exact RONS speciation on the tissues and their individual contribution to the plasma inactivation are still not fully understood. Herein, we investigated the generation and distribution of hydroxyl radical (\(\cdot\)OH), hydrogen peroxide (\(\text{H}_2\text{O}_2\)), ozone (\(\text{O}_3\)), nitrite (\(\text{NO}_2^-\)), and peroxynitrite/peroxynitrous acid (\(\text{OONO}^-/\text{ONOOH}\)) on the agarose tissue model and their contribution to yeast inactivation by helium (He) or air SMD plasma at different irradiation distances. The results show that He and air SMD plasma exhibited different RONS speciation and antimicrobial activity. The He SMD plasma mostly generated \(\cdot\)OH and \(\text{H}_2\text{O}_2\) on the tissue model, which were concentrated in every hexagon micro-discharge unit and decreased with the irradiation distance, while the air SMD plasma mainly produced \(\text{O}_3\), \(\text{NO}_2^-\), and \(\text{OONO}^-/\text{ONOOH}\), which were uniformly distributed on the whole tissue model. More importantly, the \(\cdot\)OH generation on the tissue model by the He SMD plasma was derived from the plasma delivery, while UV photolysis led to the \textit{in situ} \(\cdot\)OH generation by the air SMD plasma. Additionally, the air SMD plasma has a higher inactivation efficiency than the He SMD plasma and the major antimicrobial agent for He and the air SMD plasma is, respectively, \(\cdot\)OH and \(\text{O}_3\) in this plasma–tissue interaction system.

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discharge may be more suitable for skin disinfection. Therefore, it is necessary to investigate the RONS speciation in the SMD plasma–tissue interaction system and their individual contribution to SMD plasma sterilization.

Herein, we compared the generation and two-dimensional (2D) distribution of five representative RONS [hydroxyl radical (⋅OH), ozone (O₃), H₂O₂, NO₂⁻, and peroxynitrite/peroxynitrous acid (OONO⁻/ONOOH)] in helium (He) and air SMD plasma at different irradiation distances by using an agarose tissue model with embedded chemical reporters. Especially, we also evaluated the effects of UV photolysis on the ⋅OH generation in He and air SMD plasma. Additionally, the inactivation efficiency and killing pattern of SMD plasma against yeast cells (as a surrogate for the fungal pathogens) were measured and the Pearson correlation coefficient between the inactivation efficiency and the RONS concentration was analyzed to estimate the contribution of these RONS to yeast inactivation by He or air SMD plasma.

Figure 1(a) shows a schematic diagram of the experimental setup. The detailed information of the plasma device can be found in Ref. 20. The tissue model was made of 1% (w/v) agarose gel and its shape and size are shown in Figs. 1(b) and 1(c). Figures 1(d) and 1(e) are the photographs of He and air plasma discharge at a gas flow rate of 1.2 standard liter per minute (slm) and powered by a 5 kHz sinusoidal voltage with a peak-to-peak value of 8 kV. The air SMD displayed an obvious filamentary discharge mode and the plasma had dark areas [Figs. 1(e) and 1(g)]. Conversely, the He SMD seemed like a glow discharge and the plasma uniformly distributed over the whole dielectric surface [a little filamentary discharge was due to the residual air in He [Figs. 1(d) and 1(f)]].

The optical emission spectrum (OES) was used to investigate the major excited species generated in He and air SMD plasma. In Fig. 2(a), the spectrum of He SMD plasma was dominated by OH (A → X) band emissions (306–309 nm), N₂ second positive system [N₂ (C → B)] emissions (316 nm, 337 nm, 357 nm, and 380 nm), and He emissions (389 nm, 501 nm, 667 nm, 706 nm, and 728 nm). The OH (A → X) emissions were produced via the water dissociation caused by electrons or metastable He (He*) impact (reactions 1 and 2) (all the reactions involved in this study are listed in supplementary material S4). The N₂ (C → B) emissions were produced upon collision of the electrons or He* with the tiny residual air molecules in the chamber. For the air SMD plasma, the spectrum was dominated by the N₂ (C → B) emissions at 316 nm, 337 nm, 357 nm, 380 nm, 394 nm, 399 nm, and 405 nm as a result of the direct or stepwise electron impact excitations and the pooling reaction of the metastable N₂ [Fig. 2(b)]. Compared with the He SMD plasma, the emission intensity of N₂ (C → B) was much higher in the air SMD plasma due to the presence of much more N₂ molecules in the chamber by using air as the working gas, while the emission intensity of the N₂⁺ first negative system [N₂⁺ (B → X)] at 391 nm in the air SMD plasma was much lower, which was probably attributed to many other excitation processes to produce N₂⁺ in the He SMD plasma except electron impact, such as the charge transfer (reaction 3) and He* excitation of the ground state N₂ (reaction 4). Additionally, the emission intensity of ⋅OH in the air SMD plasma was much weaker compared with the He SMD plasma probably due to the following two reasons. One is that O₂ in the air gas can consume the free electrons by reacting with them to form O₂⁻, consequently decreasing the density of ⋅OH derived from the water dissociation by electron impact. The other is that the oxygen species such as O and O₃ formed in the air plasma can react with ⋅OH. The OES results indicated that the major excited species in the gas-phase He and air SMD plasma were, respectively, ⋅OH and excited...
N\textsubscript{2}, which can result in the different RONS speciation on the tissue model.

Given that skin tissues always contain much water and water evaporation from the skin tissues during plasma treatment could influence the RONS generation\textsuperscript{24-27}, the variation of relative humidity in the micro-environment between the tissue model and the mesh electrode during the 10 min He or air plasma discharge process at different irradiation distances was detected. In Figs. 2(c) and 2(d), the relative humidity of the He and air SMD plasma at different irradiation distances was all first increased rapidly and then remained stable, which could be attributed to the water evaporation from the tissue model. Additionally, the average relative humidity was decreased with the irradiation distance for both the He and air SMD plasma due to the reason that the shorter irradiation distance resulted in a higher temperature of the tissue model (see supplementary material S3), which could enhance the water evaporation.

Then, the 2D distribution and relative concentration of \textbullet{OH}, O\textsubscript{3}, H\textsubscript{2}O\textsubscript{2}, NO\textsuperscript{2−}, and OONO\textsuperscript{−}/ONOO\textsuperscript{−} on the tissue model after 10 min He or air SMD plasma discharge process at different irradiation distances were measured and the detailed procedures are shown in supplementary material S1. Among the five RONS, \textbullet{OH} is the most powerful oxidizing species (2.8 V), which can drastically react with almost all biomolecules\textsuperscript{19}. For the He SMD plasma, \textbullet{OH} was mainly concentrated at the center of every micro-discharge unit at short irradiation distances (1 mm, 2 mm, and 3 mm) and almost disappeared at long irradiation distances (5 mm and 10 mm) [Fig. 3(a-I)]. The \textbullet{OH} concentration was significantly decreased with the irradiation distance probably due to the following two reasons [Fig. 3(a-III)]. One is that the \textbullet{OH} has a short diffusion distance due to the short lifetime and high reactivity. The other is that the short irradiation distance has a high relative humidity, which may enhance the \textbullet{OH} generation. We also evaluated the effect of UV photolysis on the \textbullet{OH} generation via reaction 5\textsuperscript{9,28} by using...
a quartz plate covered on the tissue model, which could block the RONS diffusion and has no effects on the UV transmission. -OH cannot be observed when the tissue model was covered with a quartz plate [Fig. 3(a-II)], indicating that the -OH on the tissue model was mainly derived from the plasma delivery rather than UV photolysis. Figure 3(a-IV) describes the probable transportation pathway of -OH between the electrode and the tissue model in the He SMD plasma. For the air SMD plasma, -OH was uniformly distributed on the whole tissue model surface and its fluorescence was much weaker compared with the He plasma at short irradiation distances without quartz plate [Fig. 3(b-I)]. Moreover, -OH could still be observed when the tissue model was covered with a quartz plate [Figs. 3(b-II) and 3(b-III)], indicating that UV photolysis leads to in situ -OH generation on the tissue model in the air SMD plasma. More interestingly, the -OH concentration was slightly increased with the irradiation distance and the tissue model covered with a quartz plate had a higher -OH concentration, which can be explained in Fig. 3(b-IV). It is well known that the water molecules could consume UV, thereby decreasing the UV transmission to the target. Herein, the shorter irradiation distance had a higher relative humidity, which could attenuate the UV intensity. Conversely, the quartz plate could block the water evaporation, consequently enhancing the UV intensity.

Unlike -OH, O₃ is a long-lived RONS, which plays important roles in air plasma sterilization.₂⁶-₂⁸ In Fig. 4, almost no O₃ production can be observed in the gas-phase He plasma and tissue model, while the concentrations of gas-phase and tissue-phase O₂ in the air SMD plasma were both much higher, which was due to the fact that the air gas contains much more oxygen and thus could increase the O₂ production via reaction 6. Moreover, the O₂ was uniformly distributed on the tissue model and the irradiation distance almost had no effects on the O₂ production in the air SMD plasma due to the long diffusion distance of O₂. It is noteworthy that a slight raise of O₃ in the first 30 s in the He plasma [Fig. 4(a)] can be attributed to the residual air in the chamber. Meanwhile, the white round shape formed in the He plasma [Fig. 4(c)] was possibly caused by -OH, which can also bleach the indigo blue color.₂⁹

Besides O₂, H₂O₂ is also an important long-lived antimicrobial agent in CAP. It is generally believed that the aqueous H₂O₂ was derived from the dissolution of gaseous H₂O₂ and the combination reaction of -OH in water (reaction 7). As shown in Fig. 5(a), H₂O₂ presents a similar 2D distribution pattern and change trend to that of -OH in the He and air SMD plasma, indicating that -OH combination in the gas–tissue interface contributed to H₂O₂ generation on the tissue model. H₂O₂ can react with NO₂ to form a more bioactive species OONO⁻/ONOOH (reaction 8). In Fig. 5(b-III), the air SMD plasma had a markedly higher NO₂⁻ generation on the tissue model compared with the He SMD plasma due to the fact that NO₂⁻ are mainly derived from the dissolution of gas-phase nitrogen oxides (NOₓ) formed by the reactions of dissociated N₂ and O₂.₃⁰ The NO₂⁻ concentration in the air SMD plasma was significantly decreased with the irradiation distances except 1 mm. The reason may be that although 1 mm is the shortest delivery distance of the gas-phase NOₓ, the high relative humidity at 1 mm might have adverse effects on the NO₂⁻ generation. More interestingly, NO₂⁻ was uniformly distributed on the tissue model in the air SMD plasma, while the magenta-colored azo compounds were not generated in every micro-discharge unit at short irradiation distances in the He SMD plasma [Figs. 5(b-I) and 5(b-II)]. The reason may be that -OH formed in this area can not only oxidize NO₂⁻ to form HNO₃ (reaction 9), but also directly degrade azo dyes.₃¹ Additionally, the distribution pattern and change trend of OONO⁻/ONOOH were similar to those of NO₂⁻ in the air SMD plasma and the OONO⁻/ONOOH concentration in the air SMD plasma was also higher than that in the He SMD plasma [Fig. 5(c)].

Next, we investigated the antimicrobial effects of 10 min He or air SMD plasma against yeast cells by measuring the inactivation efficiency and killing pattern (detailed procedures are given in Figs. 4 and 5). FIG. 4. (a) The variation of gas-phase O₃ concentration and (b) average gas-phase O₃ concentration during 10 min He or air plasma discharge process at different irradiation distances. (c) The distribution of O₃ on the tissue model after 10 min He or air SMD plasma treatment at different irradiation distances. (d) The corresponding absorbance of indigo trisulfonate.
supplementary material S2). Expectedly, the killing pattern of yeast cells corresponded to the distribution of the major RONS on the tissue model. For the He SMD plasma, the colony was not formed in the presence of \( \cdot \text{OH} \) and \( \text{H}_2\text{O}_2 \) and the inactivation efficiency was decreased with the irradiation distance [Figs. 6(a) and 6(c)], whose change trend was similar to that of the \( \cdot \text{OH} \) and \( \text{H}_2\text{O}_2 \) concentrations, indicating that \( \cdot \text{OH} \) and \( \text{H}_2\text{O}_2 \) play important roles in the yeast inactivation by the He SMD plasma. For the air SMD plasma, no yeast colonies appeared on the tissue model due to the uniform distribution of the major RONS on the tissue model [Fig. 6(b)]. The inactivation efficiency was the same for all irradiation distances, which was consistent with the change trend of \( \cdot \text{OH} \), \( \text{H}_2\text{O}_2 \), and \( \text{O}_3 \) concentrations, indicating that they all contribute to the air SMD plasma sterilization.

Furthermore, in order to estimate the individual contribution of these five RONS to yeast inactivation by He or air plasma, we analyzed the Pearson correlation coefficient between the inactivation efficiency and the RONS concentration. According to the results (see supplementary material S5), the most important antimicrobial agent for the He and the air SMD plasma is, respectively, \( \cdot \text{OH} \) (0.973*) and \( \text{O}_3 \) (1.000**). Additionally, the air SMD plasma exhibited a markedly stronger antimicrobial activity compared with the He SMD plasma. The air plasma achieved a 4.5 log reduction of yeast cells, while the maximum inactivation efficiency of the He plasma was only 2 log reduction. Despite the excellent inactivation efficiency of the air SMD plasma, it may be not a good choice for skin disinfection due to the reason that the gas-phase \( \text{O}_3 \) reached a high level (about 16 ppm) during 1 min air plasma treatment, which could seriously threaten the human health. The U.S. Environmental Protection Agency (EPA) reports that exposure to ozone (0.3–2 ppm) can induce inflammatory responses in various animal models.\textsuperscript{36}

In conclusion, we compared the RONS speciation and antimicrobial effects of the He and the air SMD plasma. The He SMD plasma has a higher \( \cdot \text{OH} \) and \( \text{H}_2\text{O}_2 \) generation on the tissue model, which were mainly produced at the center of every hexagon mesh electrode and decreased with the irradiation distance, while the concentrations of \( \text{O}_3 \), \( \text{NO}_2^- \), and \( \text{OONO}^-/\text{ONOOH} \) were higher in the air SMD plasma, which were uniformly distributed on the whole tissue model. Importantly, the \( \cdot \text{OH} \) generation on the tissue model by the He and the air SMD plasma was, respectively, derived from the plasma delivery and UV photolysis. The air SMD plasma exhibited a stronger antimicrobial activity compared with the He plasma and the key antimicrobial agent for the He and the air SMD plasma is, respectively, \( \cdot \text{OH} \) and \( \text{O}_3 \). This work gives an insight into the major antimicrobial agents in the SMD plasma-tissue interaction system by using different working gases, which could promote the development of the SMD plasma applied to skin disinfection.
See the supplementary material for detection methods for OOH, O$_3$, H$_2$O$_2$, NO$_2^-$, and OONO$^-$/ONOOH (S1), microbiological analysis (S2), the temperature of the tissue model surface (S3), reactions involved in this study (S4), and Pearson correlation coefficients between the log reduction and OOH, O$_3$, H$_2$O$_2$, NO$_2^-$, and OONO$^-$/ONOOH (S5).

**AUTHOR’S CONTRIBUTIONS**

M.D. and H.X. contributed equally to this work.

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