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Bactericidal effects in water sprayed through transient spark in air and related formation of ROS and RNS

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Abstract— Chemical and bio-decontamination effects induced by plasma in water upon electro-spraying through DC-driven positive transient spark discharge in air were investigated. Inactivation of *Escherichia coli* in plasma treated water was determined in dependence on the solution pH (controlled by buffers) and correlated with chemical changes in water upon treatment by plasma. Productions of H_2O_2, nitrites, nitrates, peroxynitrites and pH changes were determined and the extent of oxidative stress induced in bacteria was evaluated by measuring the thiobarbituric acid reactive substances (TBARS). The degree of inactivation and oxidative damage of bacteria increased with increasing acidity of the solution. Acidic nitrites with H_2O_2 were determined as the most important bactericidal agents in water treated by air plasma.

Keywords—Plasma electrospray, water, bacteria, hydrogen peroxide, nitrites, acidification

I. INTRODUCTION

A great number of publications on plasma decontamination and plasma medicine appearing recently witness a quick evolution and a great potential of this new interdisciplinary field. Nowadays it is evident that cold atmospheric pressure plasmas can efficiently kill various microbes, even highly resistant forms such as bacterial spores and biofilms, and foster interesting phenomena in the cells of higher organisms leading to various therapeutic effects. The mechanisms of plasma-cell interaction are not fully understood yet, although a great research effort has been dedicated to elucidate the respective roles of various plasma agents in the interaction with live cells, such as charged particles, neutral reactive species, UV radiation, electric field and heat [1]. The most studies agree on that the bactericidal effects of atmospheric pressure cold plasmas are dominantly due to reactive neutral species (mostly radicals) and perhaps some ions, such as superoxide anion O_2^-. For many practical applications, bio-decontamination under wet conditions is important. In addition, plasma medical applications deal with cells and tissues in their naturally wet environment. When bacteria are immersed in a liquid or in a gel-like material with water content, neither ions nor electrons can interact directly with the bacteria as they are strongly absorbed by the liquid when applied through the gas–liquid interface. In these processes, plasmas induce various chemical effects leading to active species that can interact with cells in the liquid environment, either leading to bactericidal effect or various complex processes resulting in therapeutic effects in eukaryotic cells, or both.

Plasmas generated in air in contact with water are of the great interest because they produce large quantities of reactive oxygen species (ROS) and reactive nitrogen species (RNS) and seem to be the most efficient in bio-decontamination by means of plasma [2-6]. Biological and therapeutic effects of air spark-like discharges on tissues have been also demonstrated [7-9]. Even He or Ar plasma jets perform the strongest bactericidal efficacy with O_2 or air admixtures or when the rare gas plasma entrains air components [10]. Air plasma treatment of water and aqueous solutions typically leads to acidification [10-15] which can normally be explained by dissolution of NO_x species formed in the air plasma in water [6, 11, 16]. Some authors report acidification by other mechanisms, even in pure O_2 plasma by the action of O_2^- anions [14]. However, most confirm the NO_x mechanism. Acid environment itself, despite being crucial in bactericidal effect of plasma treated water, did not prove to be the main antibacterial agent. Several authors reported that addition of various acids resulting in the same pH as in the plasma treated solutions (usually 2.5-4.8) does not lead to the same bactericidal efficacy. It is acid environment in synergy with plasma agents leads to the bacterial inactivation [3, 10, 17]. However, it is not clear enough yet, which plasma agents and which ROS/RNS have dominant roles in water decontamination and what is the associated plasma-induced water chemistry. Hydrogen peroxide, nitrate and nitrite anions, as well as other species such as peroxynitrites, have been identified in plasma-activated water [2, 6, 10, 17].

In this work we investigated the chemical effects induced in water electro-sprayed through DC-driven positive transient spark discharge. We measured formations of hydrogen peroxide (H_2O_2), nitrites (NO_2^-) and nitrates (NO_3^-) and peroxynitrites ($ONOO^-$) in plasma treated water. Production of these chemical species was correlated with the pH changes and with bactericidal effects observed on *Escherichia coli,* suspended in plasma treated solutions, and with the oxidative stress induced in cell membranes of these bacteria. This work follows our previous studies of bio-decontamination of electro-sprayed water in the transient spark [18, 19].

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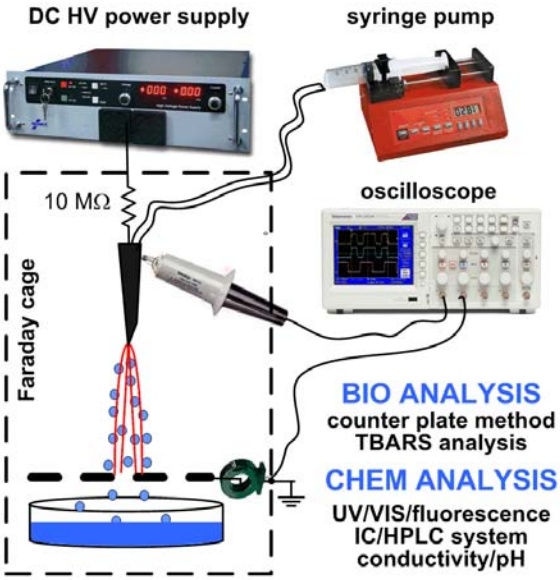

Fig. 1. The experimental setup enabling the contaminated water to be sprayed through the high voltage hollow needle electrode

Fig. 2. Photographs of the transient spark with electro-spray of water in 8 mm gap, water flow rate 0.5 mL/min, 9 kV.

II. Methodology

The experimental setup for DC-driven transient spark in point-to-plane geometry, with a high voltage (HV) hypodermic hollow needle electrode enabling water flowing through the discharge zone and a mesh electrode is depicted in Fig. 1. The inter-electrode spacing was usually kept at 10 mm. A positive DC high voltage was applied through the ballast resistor *R* (5-10 MΩ). The discharge voltage was measured by a high voltage probe Tektronix P6015A. The discharge current was measured: on a 1 Ω resistor or by a current monitor Pearson 2877. The current and voltage signals were processed by a digitizing 200 MHz oscilloscope Tektronix TDS 2024. The contaminated water flowed directly through the high voltage hollow needle electrode. The effect of electrostatic spraying occurred when the high voltage was applied on the needle electrode [18, 19]. The typical discharge visual appearance is shown in the photograph in Fig. 2.

Measurements of hydrogen peroxide and peroxynitrite formed in plasma treated water were performed by colorimetric methods using UV/VIS spectrophotometer Unicam Helios Gamma and UV/VIS/ Fluorescence microplate reader Thermo VarioScan Flash, respectively. The concentration of hydrogen peroxide was determined by the reaction of H_2O_2 with titanyl ions with the absorbance measurements at 407 nm. The concentration of peroxynitrite was determined by the reaction with 2,7-dichlorodihydrofluorescein diacetate (DCF-DA) with absorbance measurements at 500 nm and with fluorescence measurements using excitation and emission wavelengths of 502 and 523 nm, respectively (PerkinElmer LS 45, 50 Hz) [20, 21]. The concentrations of nitrites and nitrates were measured by ion chromatography using a HPLC system Shimadzu LC-10A*vp* with UV (210 nm) and suppressed conductivity detection. Analyses were made by means of a 7-µm Allsep A1 anion exchange column (10 cm × 4.6 mm) with 0.85 mmol L^{-1} $NaHCO_3$/0.9 mmol L^{-1} Na_2CO_3 as the eluent (flow rate of 1.2 mL min^{-1}). Changes of pH and electrolytic conductivity in plasma treated water were measured by pH and conductivity probes (WTW, Adwa).

Aqueous solutions of various initial pH and conductivities used for plasma treatment experiments were prepared by addition of different salts to deionized water (σ = 1 µS/cm, pH=5.5):

- NaH_2PO_4 solutions (σ = 0.6 mS/cm, pH = 5.5). These solutions were prepared to mimic the natural conductivity of tap water (~0.6 mS/cm). NaH_2PO_4 salt was used because it has similar chemical composition with the phosphate buffer described below but no buffering activity. In this work we designated these non-buffered solutions as **'water'**.
- Na_2HPO_4/KH_2PO_4 phosphate buffered (PB) solutions (σ=0.6 mS/cm, pH=6.9), designated as **'PB'**.
- physiological NaCl (saline) solutions (NaCl concentration 0.85% vol., σ = 6.35 mS/cm, pH = 6.7), non-buffered, designated as **'saline'**. Saline solution is a natural cell environment.
- physiological NaCl (saline) solutions (concentration 0.85% vol., σ = 6.0 mS/cm, pH = 6.8) with Na_2HPO_4/KH_2PO_4 phosphate buffer, designated **'PBS'**.

Bio-decontamination effects were tested on Gram-negative *Escherichia coli* (CCM3954) in water with initial populations from 10^6 to 10^8 colony forming units per mL (CFU/mL). The microbial cultivation was carried out in a sterile environment in the following steps: an overnight bacterial culture was first prepared in a shaker with sterile liquid nutrient. Cultivated bacteria in the liquid nutrient were compared with McFarland turbidity scale to assess their initial population per mL. They were then centrifuged several times and diluted in water/saline solution to obtain desired concentrations. Alternatively, bacteria (*E. coli*) pre-cultivated on gel discs were directly dissolved in the desired solution under study.

The plasma experiments with bacteria suspensions were performed with positive transient spark and repeated 10-15 times. The number of bacteria cells in the solution was assayed by counting colony forming units cultivated on agar plates (MFC, HiMedia, Mumbai, India; Biolab). Usually, 50 or 500 μL of plasma treated and reference (control) samples were used for cultivation and 3–4 Petri dishes from each sample were taken for statistical evaluation. These were incubated during 12–24 h in a thermostat at 37°C. The viability of the bacteria

was determined as the ratio of the concentration of surviving bacteria in plasma treated samples to the total concentration in reference samples.

Interaction of ROS with the bacterial cell membranes results in the peroxidation of membrane lipids. The final product of lipid peroxidation is malondialdehyde (MDA), quantifiable by colorimetric method by the reaction with thiobarbituric acid (TBA) at 90–100◦C [35]. This method of *thiobarbituric acid reactive substances* (TBARS) was applied to measure the oxidative stress induced in bacteria in water exposed to the discharge. The TBARS concentrations was determined from the absorbance of MDA measured at 532 nm (ε=1.57×10^5 L/(mol cm)) [19].

Statistical evaluation of experimental data was performed using StatsDirect and Origin software and expressed graphically in figures by medians with 25 and 75 percentiles (box), 1 and 99 percentiles (error bars), and min and max values (x symbols).

III. Results

A. Transient spark discharge (TS)

Electrical parameters and emission spectra of transient spark discharge (TS) operating in atmospheric air with water spray were documented in detail in our previous works [22, 23]. The typical voltage and current waveforms of TS discharges in 10 mm gap (with electro-spray of water) are shown in Fig. 3.

When a positive high voltage of a few kV is applied to the point electrode, streamer corona appears, As the voltage is further increased (to ~12 kV in 10 mm gap), the streamers establish a conductive channel that gradually heats, thus enhancing the reduced electric field *E/N*, which eventually leads to a spark breakdown with excessive current pulse. In our case, the spark pulse current was limited by the ballast resistor *R* that drops the voltage as the current increases, and the capacity *C* between the electrodes is small (order of 10 pF). *C* is a sum of the internal capacity of the discharge gap and the capacities of the high voltage cable and the voltage probe. Thus, when the sparks forms, it is only transient since the discharged energy is small (0.1–1 mJ per pulse) so we name it *transient spark*. *C* is recharged after the pulse, by a growing potential on the stressed electrode and triggers a new pulse. The repetitive frequency of pulses is 0.5–10 kHz, and increases with the applied voltage. Due to the very short pulse duration (~10–100 ns), the plasma cannot reach equilibrium conditions and remains at relatively cold (~ 500–1500 K), depending on frequency, i.e. dissipated power.

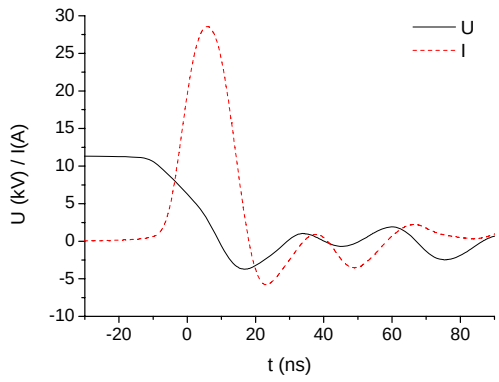

Fig. 3. Typical voltage and current waveforms of TS discharge with electro-spray of water in ns time scale, 10 mm gap.

With water electro-spray through TS, a great care was taken to keep the constant electrical parameters throughout each experiment since the water spray substantially perturbs the discharge regularity (especially jitters the pulse frequency). In order to keep the constant power dissipated into the discharge, the pulse frequency was controlled at ~1 kHz. The typical applied power used in this work was 1-2 W, with energy 1-2 mJ per pulse. All experiments were performed in positive transient spark mode. The water was sprayed through the high voltage hollow needle electrode under a constant flow rate of 0.5 mL/min.

B. Bactericidal effects of plasma treated water

Bacterial suspensions of *E. coli* (CCM3954) were dispersed in aqueous solutions and sprayed through the discharge while keeping the constant flow rate 0.5 mL/min. Fig. 4 shows the bactericidal efficiency of *E. coli* in log reduction for both non-buffered 'water' (NaH_2PO_4 solutions described above) and saline, directly after plasma treatment and 5 h later. Up to 7-log reduction in the number of bacteria was obtained in water, as well as in saline. Interestingly, when bacteria were left in the plasma treated water or saline for 4-5 h longer, a slight enhancement of the bactericidal effect was observed. This indicates the ongoing biochemical processes in the plasma activated water even after plasma treatment that were also observed in [2, 3, 14, 15, 17].

The bactericidal effect of plasma treated water and saline was always accompanied with the decrease in the solution pH (down to pH ~3) and the increase in the solution conductivity (water 600 → ~1040 μS/cm, saline solution ~6350 → ~6850 μS/cm). However, additional tests (not shown) performed to evaluate whether low pH is the main bactericidal agent showed that the nitric or hydrochloric acid solution of the same pH did not lead to the same biocidal effects. In agreement with [10, 15, 17], we confirm that rather acid environment in synergy with plasma agents leads to the bacterial inactivation.

To further elucidate the role of pH in the plasma induced bactericidal effects, experiments with phosphate buffered solutions (PB and PBS) were performed. Preliminary experiments with these two PB solutions proved their sufficient buffer capacity, i.e. their pH value remained fairly constant upon treatment by the discharge (i.e., at pH of 6.8) or decreased very little. As a consequence, the bactericidal effect was strongly reduced in PB and PBS with respect to non-buffered solutions, but not completely stopped (about 1-log bacteria reduction). Fig. 5 compares the bactericidal effect (log reduction) of water and PB obtained for *E. coli* immediately after plasma treatment and 5 h later. Interestingly, the decontamination in plasma treated PB increased substantially after 5 h, without further pH change. The effect was very similar in saline and PBS.

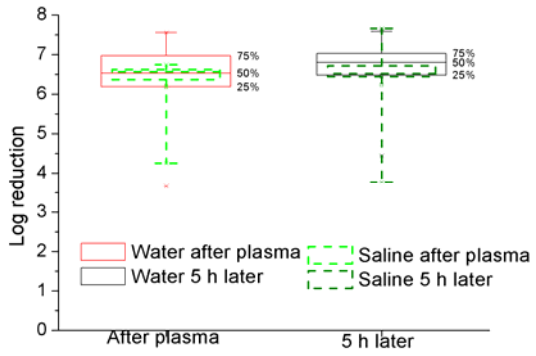

Fig. 4. *E. coli* inactivation obtained in water (σ=0.6 mS/cm) and saline (σ=6.35 mS/cm), directly after plasma treatment and 5 h later.

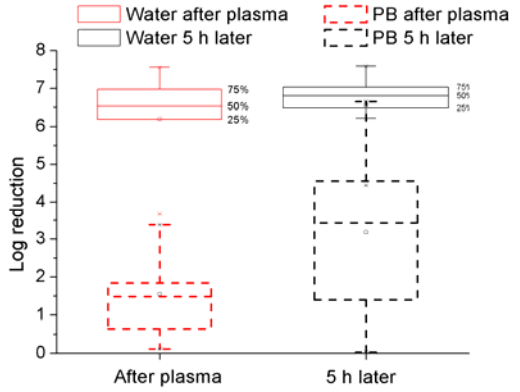

Fig. 5. *E. coli* inactivation after plasma treatment and 5 h later obtained in water (pH~3.3 after plasma and ~3.4 5 h later, respectively) and PB solutions (pH~6 and ~5.8).

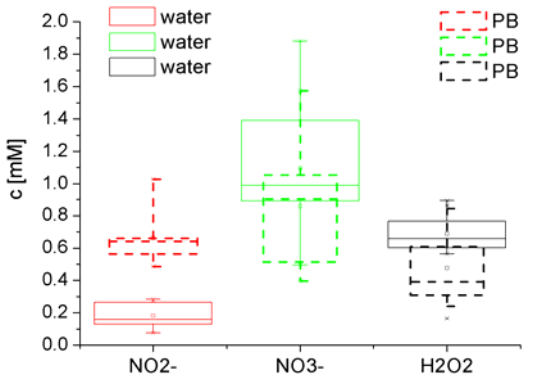

Fig. 6. Nitrite (NO_2^-), nitrate (NO_3^-) and peroxide (H_2O_2) concentrations measured in water and PB solutions after plasma treatment.

C. Chemical effects induced in water by plasma

In order to evaluate the mechanisms participating in bacterial inactivation, the chemical effects induced in water sprayed through the transient spark discharge was studied in more detail. Since the discharge operates in atmospheric humid air we focused mainly on the formation of ROS and RNS. Previous measurements made by optical emission spectroscopy of TS [22] showed a presence of atomic O, N and H, OH radicals, and the N_2^+ ions. Part of O radicals reacts with air O_2 and form ozone O_3. There was practically no UV C radiation detected from TS. In this work we measured formations of hydrogen peroxide (H_2O_2), nitrites (NO_2^-) and nitrates (NO_3^-) and peroxynitrites ($ONOO^-$) in plasma treated water.

Fig. 6 shows the concentrations of nitrites, nitrates and hydrogen peroxide obtained in water and PB solutions after spraying through the discharge. Both solutions were of the same initial solution conductivity (600 µS/cm) but of different initial pH. In water (non-buffered NaH_2PO_4 solutions), pH decreased after passing through the discharge from the initial value of 5.5 typically to about 3, while in the case of buffered PB solutions pH remained constant (~6.9). Fig. 6 shows that non-acidic environment of PB buffers resulted in much higher concentrations of nitrites (~0.6 mmol/L) and slightly lower concentrations of H_2O_2 (~0.4 mmol/L) and nitrates (~0.9 mmol/L) compared to that for non-buffered solutions, in which the concentrations of H_2O_2 were ~ 0.7 mmol/L, nitrites ~ 0.2 mmol/L and nitrates ~ 1 mmol/L.

Such a significant pH effect on the concentrations of nitrites is result of disproportionation of nitrites into nitrates occurring under acidic conditions (Eq. 1).

$$3\ NO_2^- + 3H^+ \rightarrow 2\ NO + NO_3^- + H_3O^+ \qquad \text{(Eq. 1)}$$

This route is greatly accelerated at pH below ~ 3.5 which correlates with acid-base properties of nitrites (pK_a = 3.3). Since the only difference between buffered and non-buffered solutions was pH, it is reasonable to assume that the initial production rates of nitrites and nitrates by the discharge were the same under both conditions. The mechanism of their formation in water is the result of dissolution of NO_x formed in air plasma by gas-phase reactions of dissociated N_2 and O_2. Along with formation of NO_2^- and NO_3^- in the plasma treated water, dissolution of nitrogen oxides in water leads to the decrease of pH (Eqs. 2,3).

$$NO_2 + NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2\ H^+ \qquad \text{(Eq. 2)}$$
$$NO + NO_2 + H_2O \rightarrow 2\ NO_2^- + 2\ H^+ \qquad \text{(Eq. 3)}$$

The difference in the concentrations of NO_2^- and NO_3^- measured in both solutions is then a result of subsequent post-discharge reactions occurring in plasma treated water leading to disproportionation of nitrites into nitrates (Eq. 1).

The formation of nitrates may also proceed via the liquid-phase reaction of NO_2 with OH radical to form peroxinitrous acid (ONOOH) or its conjugate base peroxynitrite ($O{=}NOO^-$), which subsequently decay into NO_3^-. Or in the presence of hydrogen peroxide, peroxynitrite can be also formed by the reaction of nitrite anion with H_2O_2.

$$NO_2^- + H_2O_2 \rightarrow O{=}NOO^- + H_2O \qquad \text{(Eq. 4)}$$

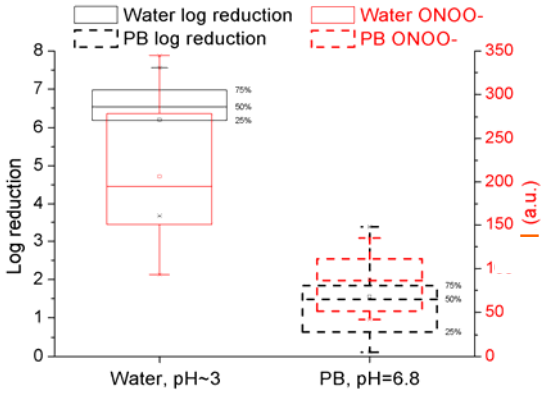

Fig. 7. Bactericidal effect (log reduction) of water and PB solutions, after plasma treatment correlated with the $ONOO^-$ concentrations, I is a fluorescence emission at 523 nm with reference subtracted.

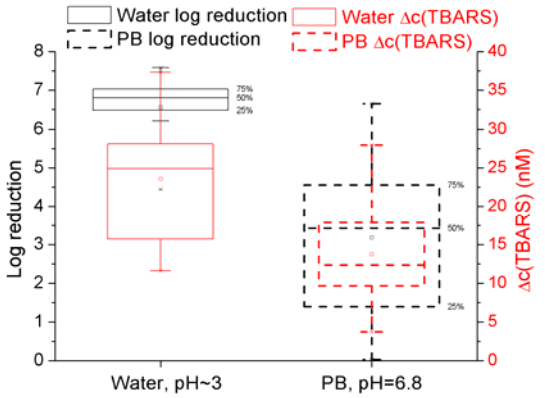

Fig. 8. Bactericidal effect (log reduction) of plasma treated water and PB solutions, 5 h after plasma treatment correlated with the TBARS concentrations, Δc(TBARS) means with reference concentrations subtracted.

This route occurs under acidic conditions and might be responsible for lower concentrations of hydrogen peroxide determined in non-buffered solutions of NaH_2PO_4 (water). Peroxynitrites are relatively strong oxidants with a large bactericidal effect and they may significantly contribute to inactivation process induced by air plasma in water. Under acidic conditions their bactericidal effect is determined mainly by OH radicals formed by the decomposition reaction of peroxynitrous acid [24]. It is likely that with the increasing acidity of the plasma treated solution, the contribution of peroxynitrites to bactericidal effects of plasma increased. Therefore, the role of peroxynitrites should likely increase with the increasing acidity of the plasma treated solutions. The attempt was made to correlate the concentrations of nitrites and hydrogen peroxide with the concentrations of peroxynitrites formed in plasma treated water. Since peroxynitrites are highly reactive under acidic conditions, their lifetime is very short and their detection in plasma treated water was possible only qualitative with concentrations estimated in the order of tens of µmol/L. Nevertheless, Fig. 7 shows that the relative amounts of peroxynitrites $ONOO^-$ measured by fluorescence spectroscopy correlate directly with the bactericidal effect. These qualitative measurements indicate an important role of $ONOO^-$ in bio-decontamination and their possible relation to peroxidation of cell membranes.

The results shown in Fig. 7 demonstrate that the decrease in concentration of H_2O_2 was lower than of nitrites in water (non-buffered solutions). This indicates that relative concentrations of $ONOO^-$ do not correlate perfectly with nitrites and peroxides and the main reason for lower concentrations of nitrites is more likely their acidic disproportionation (Eq. 1). In addition to Eq. 4, peroxynitrites might be also formed by other pathways such as by the reaction of nitric oxide and superoxide anion radical (Eq. 5) or by the reaction of NO_2 and OH radicals (Eq. 6). However, due to the fast decay of these radicals, these two reactions might take place only directly in the plasma zone and the formation of peroxynitrite by these routes cannot be determined by the analytical method used in this work.

$$O_2^- + NO \rightarrow O{=}NOO^- \quad \text{(Eq. 5)}$$

$$NO_2 + OH \rightarrow O{=}NOO^- + H^+ \quad \text{(Eq. 6)}$$

It should be noted, however, that nitrites are rapidly oxidized to nitrates and oxygen in the presence of ozone, thereby eliminating their content in water (Eq. 7).

$$NO_2^- + O_3 \rightarrow NO_3^- + O_2 \quad \text{(Eq. 7)}$$

At the same time, the antimicrobial properties of nitrites under acidic conditions have to be considered. These so-called “acidified nitrites” are capable of great effect on bacteria [2, 24]. In fact, it seems more reasonable to assume that acidified nitrites are more important route of bactericidal effects then through their reaction with H_2O_2 upon formation of peroxynitrites (Eq. 4).

D. Measurements of the oxidative stress

Interaction of ROS with the bacterial cell membranes results in the peroxidation of membrane lipids. The final product of lipid peroxidation is malondialdehyde (MDA), quantifiable by spectrophotometry by TBARS technique. Concentration of TBARS products were evaluated in all tested solutions (water, PB, saline, PBS) after plasma treatment of the same samples evaluated for bactericidal effect. Fig. 8 demonstrates a correlation of measured concentration of TBARS products with the bactericidal efficacy. Log reduction is shown after 5 hours post plasma treatment because TBARS method also required at least 5 hour delay post treatment. A similar correlation was observed with peroxynitrites (Fig. 7). Oxidative stress is apparently lower in the buffered solutions, which agrees with the bactericidal effect. This correlation confirms our previous results [18, 19] and indicates that cell membrane peroxidation by ROS is an important mechanism in plasma bio-decontamination.

V. Conclusion

We investigated water or saline solution electrospray treatment through the cold air plasma of transient spark. The treatment lead to acidification and the production of

nitrites, nitrates, peroxides and peroxynitrites. At lowered pH, nitrites are quickly oxidized to nitrates with peroxides and this is associated with the strong bactericidal effect tested on *E. coli*. At neutral pH in buffered solutions, nitrites are less oxidized and the biocidal effect is weaker. The bactericidal effect correlates with the amount of the formed peroxynitrites, as well as with the oxidative stress induced in cell membranes measured by TBARS method. It seems that that the interacting nitrites and peroxides in acidic conditions are the dominant bactericidal agents in water sprayed through air plasma.

ACKNOWLEDGMENT

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