6th Central European Symposium on Plasma Chemistry
Bressanone, Italy, September 6-10, 2015

Scientific Program & Book of abstracts

ISBN: 978-88-6938-045-7

CESPC-6, 6th Central European Symposium on Plasma Chemistry, Bressanone, Italy, September 6-10, 2015. Scientific Program and Book of Abstracts

Edited by Cristina Paradisi, Ester Marotta

Università degli Studi di Padova
via 8 Febbraio 2, Padova
www.padovauniversitypress.it

ISBN 978-88-6938-045-7

Chemical and Biological Effects of Air DC Transient Spark Discharge in Water Electrode System

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Abstract. In this study, the DC driven self-pulsing transient spark (TS) discharge was generated at atmospheric pressure air in contact with various water solutions. The chemical effects in water and biological effects on prokaryotic and eukaryotic cells, and biomolecules in water were investigated. Concentrations of H_2O_2 and NO_2^- in water solutions exposed to plasma were evaluated by UV-VIS spectroscopy. Biocidal effects of the plasma were tested on gram-negative bacteria *Escherichia coli* and correlated with chemical changes in the solutions. The results indicated strong bactericidal effects, more intense in non-buffered (3-4 logs) than in buffered (1-2 logs) solutions. Cytotoxic effect of the plasma was tested on eukaryotic cells, both normal Vero and cancerous HeLa. Direct treatment of cells by plasma was compared with indirect treatment of cell by plasma activated gas flow, for which much smaller cytotoxic effect was observed. The tests of viability and apoptotic behavior of cells showed persistent cytotoxic effect on HeLa cells, while in case of Vero cells the effect decreased by time. We also investigated the DNA fragmentation and protein denaturation. The results successfully demonstrated a potential of the self-pulsing TS discharge as an efficient tool for biomedical applications.

1. Introduction

Low temperature plasma generated by electrical discharges in atmospheric pressure air is the source of various reactive species, free radicals and charged particles. If the plasma is generated in a contact with liquids the active species formed in the gas-phase can initiate subsequent chemical reactions in liquid leading to various chemical and biological effects. The low-temperature plasma induced these effects at room temperatures. Therefore it has a great potential for sterilization and disinfection of termosensitive materials. With respect to biomedical applications the plasma is the most promising for blood coagulation, dental care, wound and ulcer healing, or even cancer treatment [1-4]. Despite many positive effects reported, the plasma interaction with cells remains still relatively not well understood. Therefore in this study we investigated the interaction of low-temperature plasma with water solutions containing bacteria, normal cells, cancerous cells and biomolecules. We analyzed the plasma effects from chemical and biological point of views and we compared direct and indirect plasma exposure.

2. Experimental

The low temperature plasma was generated by transient discharge in air at atmospheric pressure in two configurations allowing either direct or indirect treatment of water solutions. Direct treatment system in water electrode system (WS) depicted in Fig. 1a, was designed to provide repetitive direct contact of water solution with plasma generated by the discharge. It consisted of high voltage hollow needle electrode placed above the inclined grounded electrode in point to plane geometry with inter-electrode distance 1 cm. The electrodes were enclosed in a small chamber (volume ~ 12.5 mL) to be able to vary a gas composition (gas flow rate ~ 2 L/min). The treated water solution was supplied via a narrow channel on a grounded electrode into a laboratory tube and circulated by a peristaltic pump with variable flow rates. Effect of the direct treatment was compared to the indirect treatment, where the

solution was exposed to plasma activated gas flow of neutral species. Experimental setup of the indirect treatment system is depicted in Fig. 1b. The treated water solution was placed under the grounded mesh electrode, above which was hollow needle serving as a high voltage electrode. The gas with the flow rate 0.5 mL/min (30% O_2 in N_2) flown through the needle. In both direct and indirect systems self pulsing transient spark (TS) discharge of positive polarity generated in air at atmospheric pressure was used as a low temperature plasma source [5]. The TS discharge was driven by DC power supply connected through the ballast resistor 10 Mq to the reactor system. The electrical characteristic of the TS discharges were monitored by high voltage probe (*Tektronix P6015A*) and Rogowski type current probe (*Pearson Electronics*) connected to an oscilloscope (*Tektronix TDS 1012*).

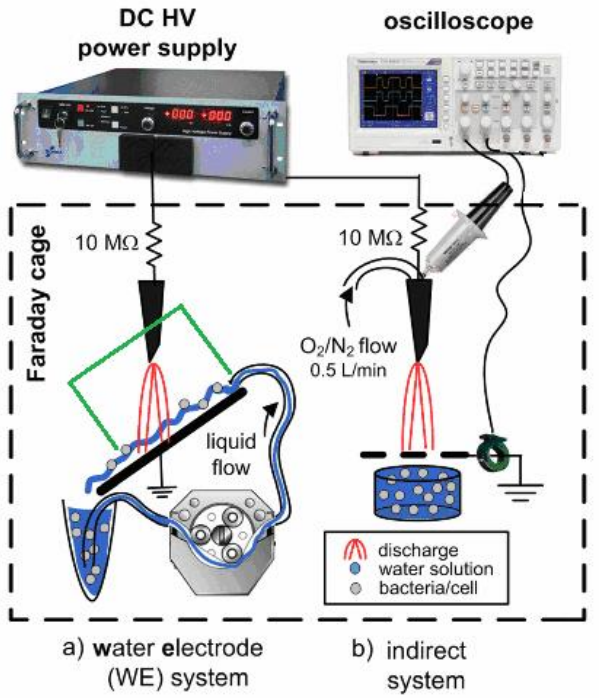

Fig. 1. Scheme of the experimental systems for a) direct and b) indirect treatment.

Chemical effects

To understand the plasma effect on living cells, it was crucial to analyze chemical changes in water solution caused by the plasma treatment. Four different water solutions with various initial pH and conductivity were subjected to plasma treatment (Tab. 1). Each solution was prepared by the dissolution of salts in deionized water (pH 5.5, ů ~2 ε S/cm). We used monosodium dihydrogen phosphate NaH_2PO_4 to mimic conductivity of tap water (*W*) however with known chemical composition. To understand the effect of pH we compared it with phosphate buffer solution (*PB*). As a physiological environment we treated also saline solution (*S*) and its buffer counter part (*PBS*). We measured concentrations of selected reactive oxygen and nitrogen species by colorimetric methods using UV/VIS absorption spectroscopy. For measurement of hydrogen peroxide H_2O_2 we utilized its reaction with titanyl ions of titanium oxysulfate $TiOSO_4$ resulting into formation of yellow-colored product with maximum absorbance peak at 407 nm. The 200 ε L of treated sample was immediately after the treatment stabilized by 30 ε L sodium azide NaN_3 to prevent the reaction of H_2O_2 with NO_2^-, and then we added 100 ε L of $TiOSO_4$. For measurement of nitrite NO_2^- we employed commercial kit (*Cayman Chemicals*) that uses Griess reagents to form pink-coloured azo-product with maximum absorbance peak at 540 nm after 10 minutes of incubation. We also monitored the pH (*WTW 3110*) and conductivity (*Greisinger Electronic GMH 3430*) of treated solution. The volume of solution was 5 mL for direct treatment, while for indirect treatment

only 100 ε L was used due to much smaller chemical effect expected.

Biological effects

Plasma induced chemistry in water solutions and formation of various reactive species in the solutions affects also the living organisms. We used different types of cells: prokaryotic, healthy eukaryotic and carcinoma eukaryotic cells. The reason for using various types of cells was to explore their different behavior under plasma induced stress. As a prokaryotic organism we used Gram-negative bacteria *Escherichia coli* (CCM3954), well-know and often used model microorganism, in a planktonic form (bacteria floating in water suspension) with initial concentration 10^7 CFU/mL. We worked in sterile conditions and according standard microbiological methods. The viability of bacteria after plasma treatment was evaluated by colony counting method and compared with the reference sample supplied through the system without a discharge. As eukaryotic representatives we selected mammalian cells, namely monkey kidney epithelial Vero cells and human cervix epithelial carcinoma HeLa cells, in order to compare healthy and carcinoma cells. The viability of cells after plasma treatment was evaluated by trypan blue exclusion test. Initial concentration of the cells in 5 mL PBS solution was $5x10^5$ cells/mL. We also investigated the effect of TS discharge on biomolecules, such as dsDNA (double strand deoxyribonucleic acid) and representative protein BSA (bovine serum albumin) in order to better understand processes that may happen inside the cell. DNA damage and BSA conformation changes were quantified by UV absorption and fluorescence spectroscopy.

3. Results and Discussion

The chemical effect on water solutions and biological effect on various targets (*E. coli*, Vero, HeLa cells, DNA and BSA) caused by TS discharge were investigated. The typical amplitude of the applied voltage was 12 ï 13 kV, amplitude and frequency of the discharge current pulses were 5 ï 18 A and 1.5 ï 4 kHz, respectively. The typical average discharge power was 1-4 W with the energy of 1-4 mJ per pulse. The characteristic voltage and current waveforms of TS are depicted in Fig. 2. We tried to keep constant electrical parameters during every experiment.

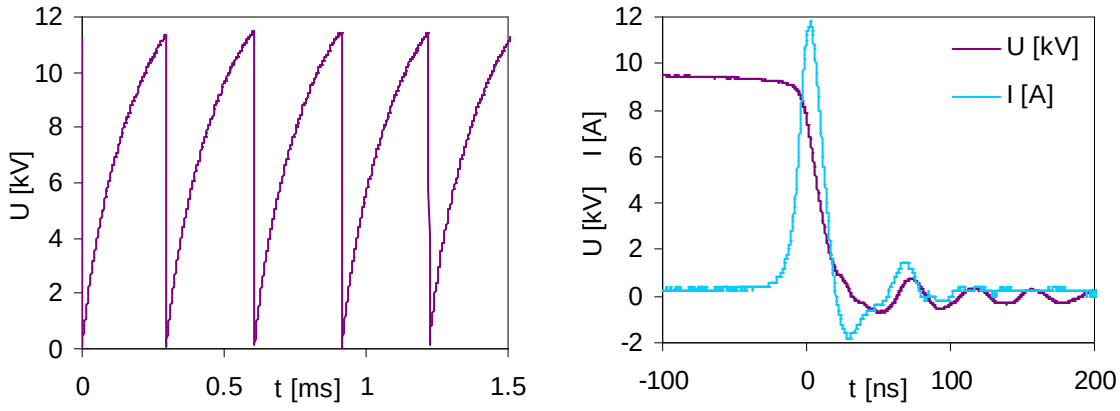

Fig. 2. Characteristic voltage and current waveforms of TS discharge in air at atmospheric pressure

Chemical effect induced by direct TS treatment in four different water solutions is described further. We treated two main water solutions (*W* and *S*) and their buffered counterparts (*PB* and *PBS*) to see the pH impact. Table 1 presents the pH and conductivity of these solutions before (initial) and after 10 minutes of direct TS treatment in the WS system.

Table 1. pH and conductivity of water solutions before and after 10 minutes of direct treatment (sample volume 5 mL, liquid flow rate 14 mL/min, applied voltage 12-13 kV, frequency ~1-2 kHz)

Water solution	**abbreviation**	**initial**		**final**	
		pH	**conductivity ů [mS/cm]**	**pH**	**conductivity ů [mS/cm]**
NaH_2PO_4	**W** - ówaterô	5	0.6	3.2	0.8
Na_2HPO_4/KH_2PO_4	**PB** - phosphate buffer	7	0.55	6.3	0.6
NaCl	**S** - saline	6.7	6	3.2	6.23
$NaCl+Na_2HPO_4/KH_2PO_4$	**PBS** ï buffered saline	7	5.9	6	6

Decrease of pH and increase of conductivity is caused by the chemical effects induced by the plasma and formation of various species in air and also in water solutions. Generating the TS discharge in contact with water at first leads to formation of active species and radicals in gas phase, mostly hydroxyl radical ^{Å}OH and nitrogen oxides NO_x. These species then dissolve in water and form hydrogen peroxide H_2O_2 and $NO_2Ƶ$ and $NO_3Ƶ$ that are responsible for the acidification of the solution and accompanied by increase of solution conductivity. Type and concentration of reactive species formed in gas phase depend on a gas mixture, in which is the discharge generated. The reactive species formed in gas phase therefore influence the reactive species subsequently formed in water solutions. Therefore we also investigated generation of the TS in various O_2/N_2 gas mixtures, measured the concentrations of H_2O_2 and $NO_2Ƶ$ in water solutions and correlated them with the biocidal effect on *E. coli*. Figures 3 and 4 shows the concentrations of H_2O_2 and $NO_2Ƶ$ in water solutions after the direct treatment in ambient air and various N_2/O_2 mixtures, respectively. In acidic conditions in non-buffered solutions (*W*, *S*) $NO_2Ƶ$ can further react and forms $NO_3Ƶ$, however in buffered solution this reaction is impossible and $NO_2Ƶ$ is accumulating. Therefore the concentration of $NO_2Ƶ$ is higher in *PB* than in *W* (Fig. 3a and 4b). In *S* and *PBS* solutions we did not measure the concentration of $NO_2Ƶ$ because of contraindication of the used analytical method with chlorine. The concentration of H_2O_2 (Fig. 3b) was higher in *W* and *S*, than in *PB* and *PBS*, because of less formation or higher rate of degradation of H_2O_2 in pH stabilized solutions. Figure 4 shows the concentrations of H_2O_2 and $NO_2Ƶ$ after 5 min treatment by TS generated in O_2/N_2 mixtures. If the discharge was generated in pure oxygen with minimum impurities, NO_2^- was not formed, its concentration was negligible and pH of the solution remained constant. On the other hand, the concentration of H_2O_2 formed in pure oxygen was very high. It is caused by more intense formation of hydroxyl radicals in gas phase that forms H_2O_2 in water, and also due to lack of NO_2^- that could potentially react with it. In nitrogen, the discharge produced only a small amount of NO_2^- due to lack of oxygen, and therefore only a small decrease of pH was observed. Compared to pure nitrogen and oxygen, in O_2/N_2 atmosphere the concentration of NO_2^- was found the highest, while the concentration of H_2O_2 was found the lowest. The absolute concentrations of H_2O_2 and NO_2^- and their relative ratio are probably important for on-going liquid chemistry. The synergetic effect of H_2O_2 and NO_2^- in acidic conditions can lead to formation of peroxinitrites and is responsible for overall strong bactericidal effect.

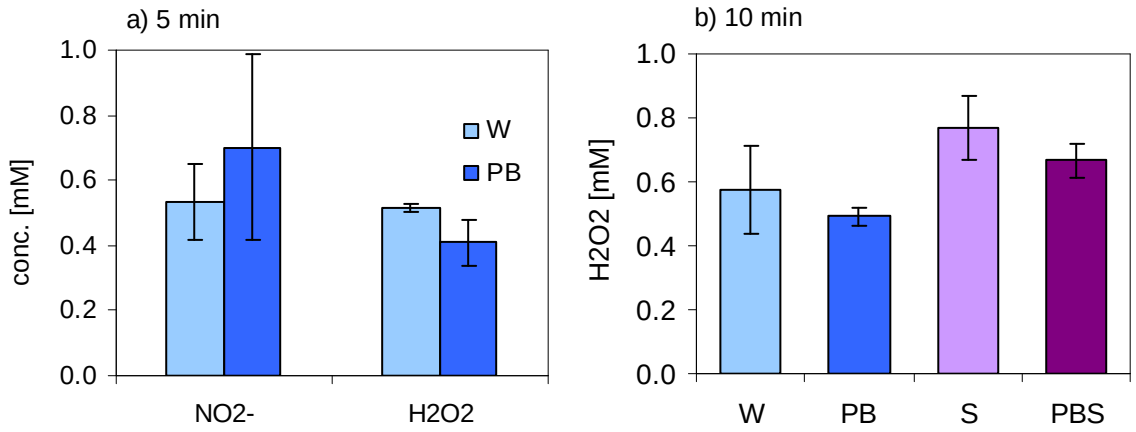

Fig. 3. Concentrations of (a) H_2O_2 and NO_2^- in *W* and *PB* solutions generated by the TS in ambient air (5 min direct treatment, sample volume 5 mL, liquid flow rate 14 mL/min, applied voltage 17 kV, frequency ~ 2 kHz). Concentrations of (b) H2O2 in buffered and non-buffered solutions (10 min direct treatment, sample volume 5 mL, liquid flow rate 14 mL/min, applied voltage 13kV, frequency ~ 1 kHz).

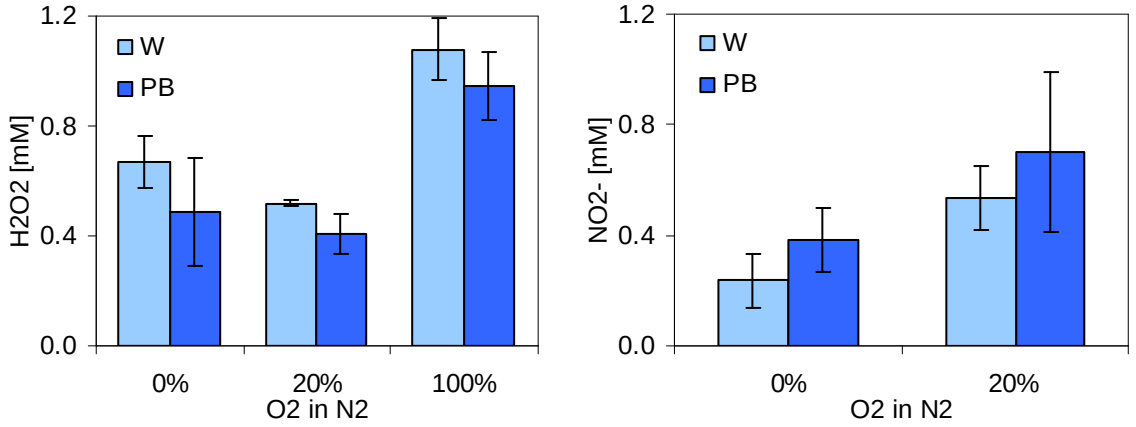

Fig. 4. Concentrations of H_2O_2 and NO_2^- in *W* and *PB* generated by the TS in O_2/N_2 gas mixtures (5 min direct treatment, sample volume 5 mL, liquid flow rate 14 mL/min, gas flow rate 2 mL/min, applied voltage 17 kV, frequency 1-3 kHz)

Bactericidal effect was tested on Gram-negative bacteria *E. coli*. Bacteria were floating in non-buffered and buffered solutions with initial concentration around 10^7 CFU/mL (colony forming unit per milliliter). In Figure 5a are depicted concentrations of *E. coli* after 10 min direct treatment in different solutions. In non-buffered solutions the decrease in bacterial population by ~ 3-5 log, while in buffered solution only ~1-2 log were observed. The stronger effect observed in non-buffered solutions can be explained by different plasma induced chemistry. In saline solution the decrease of bacterial population is most profound, while the H_2O_2 concentration is the highest. H_2O_2 induces chemical reactions forming the most reactive radical, hydroxyl radical $^{\bullet}OH$ and can damage the cell membranes by peroxidation of their lipids. However, the H_2O_2 alone cannot cause equally strong bacterial inactivation, but also other active species are responsible, especially NO_2^-. In Figure 5b the effect of gas mixture on bacteria is shown. Here again obvious contrast between buffered and non-buffered solutions can be found. The bacterial inactivation is most efficient in air like mixture, probably due to the synergetic effect of H_2O_2 and the highest concentration of NO_2^- associated with pH decrease.

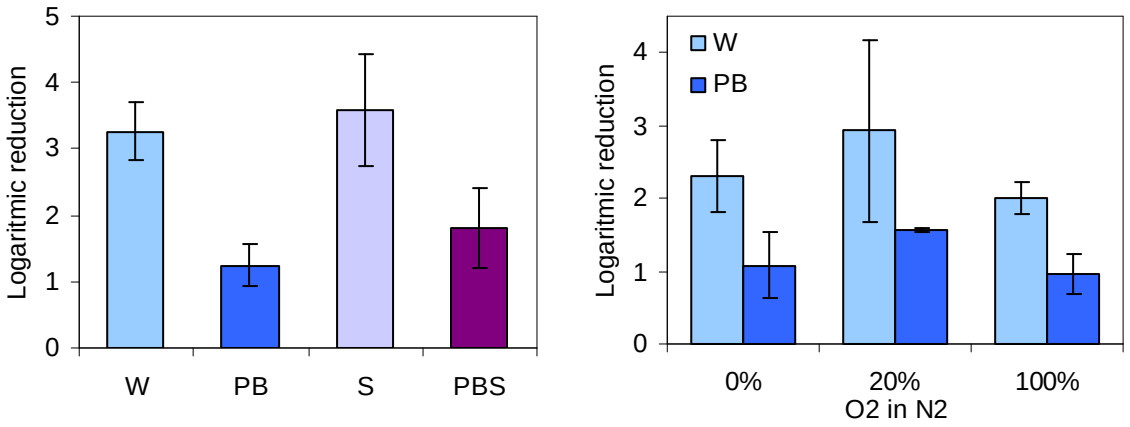

Fig. 5. Logaritmic reduction of *E.coli* population in various solutions in (a) ambient air (treatment time 10 min, liquid flow rate 14 mL/min, applied voltage 13 kV, frequency ~1-2 kHz) and in (b) various synthetic gases (treatment time 5 min, liquid flow rate 14 mL/min, applied voltage 17 kV, frequency ~1-3 kHz).

We also evaluated plasma effect on eukaryotic cells by assessing the cell viability and apoptosis. In all experiments cell viability decreased with increasing treatment time and discharge power. Cytotoxic effect of ~50% was achieved after 10 min of direct treatment induced by TS in 5 mL PBS solution of HeLa cells (Fig. 6). In comparison, indirect treatment effect on Vero and HeLa cells in 100 uL PBS was much smaller. For indirect treatment (Fig. 7), the viability of HeLa cells decreased after 2 min treatment by ~20% and remained stable after following 24 hours incubation. The viability of Vero cells also decrease by ~20% after 2 min of indirect treatment, however after 24 hour of incubation they recover to nearly ~90%. This confirms the fact that cancerous cells are more sensitive to plasma treatment and can be selectively targeted, while normal cells are more likely to be resistant and easily repaired. The experiments suggested that cells treated by plasma are more likely to die through process of apoptosis cycle (programmed silent cells death) and that plasma can also affects the cell cycle [6]. However, we have to keep in mind there may be differences in behavior of cells in real organism and those separated in a solution.

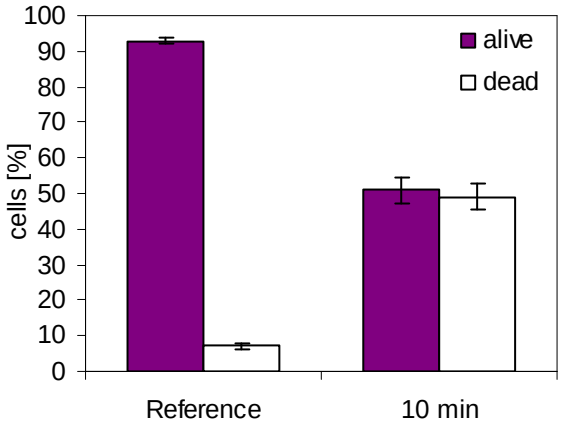

Fig. 6. Alive and dead HeLa cells after 10 minutes of direct treatment to the TS discharge in the WS system (24 hours incubation, sample volume 5 mL, liquid flow rate 14 mL/min, applied voltage 13 kV, frequency ~1-2 kHz)

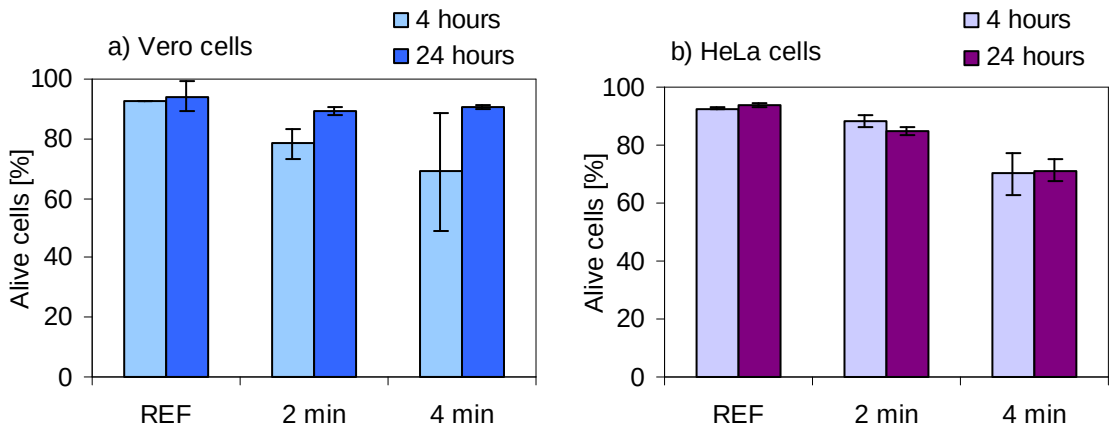

Fig. 7. Viability of HeLa and Vero cells after 2 and 4 minutes of indirect treatment to the TS discharge afterglow after 4 and 24 hours of incubation (sample volume 100 ε L, applied voltage12.5 kV, frequency ~2 kHz)

The effect of plasma treatment on biomolecules was briefly examined, too. Figure 8 shows the effects of TS on DNA. Effect on DNA was evaluated by UV absorption spectroscopy by measuring absorbance at 260 nm confirmed by fluorometric analysis of 1140 bp amplicons. The increase of DNA concentration in the figure corresponds is due to the increase of fragmentation of DNA strand and was evaluated based on increase of absorbance. We also evaluated the degradation of BSA, through fluorescence (excitation wavelength 280 nm and fluorescence maximum 345 nm). With increasing applied voltage and treatment time the fluorescence of BSA was decreasing due to protein denaturation.

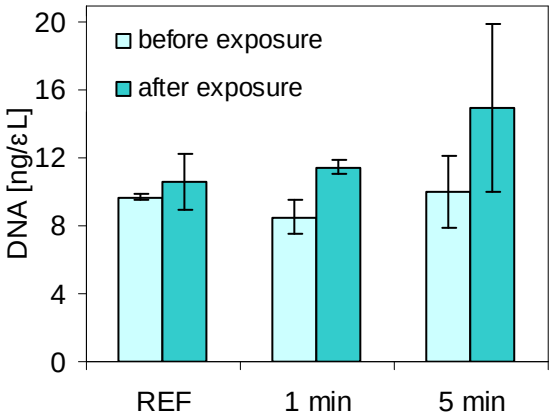

Fig. 8. Effect on DNA direct treatment (9 ng/ε L of DNA in 2 mL of deionized water, sample voume 2 mL, liquid flow rate 5 mL/min, applied voltage 15 kV, frequency ~1 kHz)

4. Conclusion

The DC driven self-pulsing TS discharge was generated at atmospheric pressure air in contact with various water solutions. We observed chemical changes in pH, conductivity and concentration of H_2O_2 and NO_2Z In non-buffered solutions was pH decrease and conductivity increase stronger than in buffered solutions. The concentration of H_2O_2 reached ~0.5 mM in *W* while in *PB* ~0.4 mM. In buffered solution were NO_2Z accumulating up to ~1 mM. According pH and H_2O_2 changes the inactivation of *E. coli* in various solutions were showed. Direct treatment of biological structures by TS discharge was compared to indirect treatment by TS activated gas flow. Total cytotoxic effect of direct treatment was much stronger in comparison with indirect treatment. After 10 minutes of direct treatment ~50% of HeLa cells were killed, while after 4 minutes of indirect treatment only ~25% were found dead. Further, we observed that normal Vero cells had ability to recover after TS treatment. The cell components DNA

and representative protein BSA were found damaged after direct treatment increasing with treatment time and applied voltage. These results demonstrated a potential of TS discharge for biomedical applications with awareness of need of further research.

Acknowledgements: The work was supported by Slovak Research and Development Agency SK-RO-0024-12 and APVV 0134-12 grants, and partially also by Slovak Grant Agency VEGA 1/0918/15 grant.

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