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The effect of a plasma needle on bacteria in planktonic samples and on peripheral blood mesenchymal stem cells

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Abstract. In this paper, we study the application of a plasma needle to induce necrosis in planktonic samples containing a single breed of bacteria. Two different types of bacteria, *Staphylococcus aureus* (ATCC 25923) and *Escherichia coli* (ATCC 25922), were covered in this study. In all experiments with bacteria, the samples were liquid suspensions of several different concentrations of bacteria prepared according to the McFarland standard. The second system studied in this paper was human peripheral blood mesenchymal stem cells (hPB-MSC). In the case of hPB-MSC, two sets of experiments were performed: when cells were covered with a certain amount of liquid (indirect) and when the cell sample was in direct contact with the plasma.

Most importantly, the study is made with the aim to see the effects when the living cells are in a liquid medium, which normally acts as protection against the many agents that may be released by plasmas. It was found that a good effect may be expected for a wide range of initial cell densities and operating conditions causing destruction of several orders of magnitude even under the protection of a liquid. It was established independently that a temperature increase could not affect the cells under the conditions of our experiment, so the effect could

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originate only from the active species produced by the plasma. In the case of those hPB-MSC that were not protected by a liquid, gas flow proved to produce a considerable effect, presumably due to poor adhesion of the cells, but in a liquid the effect was only due to the plasma. Further optimization of the operation may be attempted, opening up the possibility of localized *in vivo* sterilization.

Contents

1. Introduction

One of the leading techniques for material engineering is plasma treatment, irreplaceable in the fabrication of semiconductor devices, integrated circuits, optical devices and solar cells. The possibilities of plasma treatment surely do not end here. The constantly growing field of biomedical applications is a new frontier that drives the field [1, 2]. Sterilization of medical equipment and treatment of wounds and dental caries are only segments of that field, and they show the breadth of potential applications. The desire to use plasma for *in vivo* treatments has made several requirements for plasma sources to be met. It is obvious that for this kind of application, a plasma has to operate at atmospheric pressure. On the positive side, no expensive vacuum systems are needed; on the other hand, it is much more difficult to achieve a non-equilibrium (non-thermal) mode of operation, which is equally essential. The heat sensitivity of biomedical samples narrows the choice to non-thermal plasmas. There are many types of plasmas that can be generated under ambient pressure and temperature conditions; the need for precise and localized treatments qualifies the plasma needle as a good candidate [3].

The path to understanding the interaction between plasma and living tissues is a long one. A lot of effort has been invested in understanding the details of the basic mechanisms of this interaction. Aspects of plasma-generated heat [4], UV radiation, radicals and charge particle interactions with different kinds of bacteria and cells have been studied [5]–[15]. Nevertheless, many of the questions remain open.

Mass spectrometry of plasma generated by the needle, as well as derivative probe measurements of power delivered to it, has already been presented in our previous work [16]–[18]. The plasma needle generates radicals like N, O, O_3 and especially NO, which

plays an important role in cell metabolism. Measurements of the power delivered to the plasma itself as a parameter provides the information needed to control the heating of the target and enables us to repeat treatments with the same or very similar plasmas every time. This is much more difficult to achieve if power is measured directly at the power source, so one needs to perform direct measurements of the local heating in that case [4].

The wide potential usage of plasma technology in the biomedical sciences, besides the plasma requirements, also directs the biological effect testing. To investigate the possible applications and the ways in which the plasma needle can affect various kinds of cells in this study, we performed experiments on two model cell systems. The first one was treatment of *Escherichia coli* and *Staphylococcus aureus* bacteria in order to study the deactivation of harmful bacteria, since the plasma needle can be applied in the treatment of light bacterial infections, such as *in vivo* sterilization of skin and dental cavities. For other potential applications, including the high-precision removal of pathological cells or tissues (cancer, peeling and removal of scars), but without excessive damage to the body, or the improvement of wound healing by controlling cell adhesion, we analyzed the plasma interaction with normal, living cells. For these experiments, we have used human peripheral blood mesenchymal stem cells (hPB-MSC) as a model system to predict the degree of possible damage to the cell responses. The plasma needle has been used to induce the killing of *Streptococcus mutans* and *E. coli* bacteria [19, 20]. Its application for the sterilization of bacteria inside the tooth to cure caries without conventional mechanical preparation and loss of tooth structure has been proposed and studied [4]. *S. aureus* (ATCC 25923) has been a subject of OAUGDP plasma treatment [21] as well as of other types of plasma sources [8, 9, 22]. *E. coli* (ATCC 25922) has been used in numerous studies, and here we mention only a few [7, 9, 20], [22]–[26].

On the other hand, the plasma needle has been used to induce apoptosis and necrosis of cultured eukaryotic cells [27], rat aortic smooth muscle cells, bovine aortic endothelial cells [28] and human (epithelial cells—MR65 cells originating from non-small cell lung carcinoma [29]) tissues. Studies of the plasma treatment of human mesenchymal stem cells (MSCs) have not been performed before, and only recently we heard of the first attempt [30]. Adult MSCs are defined as multipotent cells able to differentiate into various types of end-stage, specialized mesenchymal cells, such as osteoblasts, chondrocytes, adipocytes, tenocytes and others [31]. These cells are located in and around different organs and tissues of the body. The possibility of growing these cells and their progeny in cultures offers a shortcut to test the toxicity of various chemical or physical agents on various cells and tissues of the adult organism [32, 33].

In this paper, we will present the results of plasma needle treatments of two types of bacteria. Under normal circumstances, bacteria would most likely be present either in a liquid or as a part of a biofilm. We have treated samples that were in the form of a suspension with different concentrations of bacteria. Changes in bacteria concentration after the treatments will be presented. We covered one example of Gram-positive and one of Gram-negative bacteria. The second system that was studied was mesenchymal cells (hPB-MSC). These samples were treated with and without the liquid environment. Results of the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) viability and Crystal Violet (CV) adhesion tests performed with hPB-MSC will be presented. These represent the first step on the route to developing, optimizing, understanding and using the plasma needle for the *in vivo* treatment of periodontal pockets.
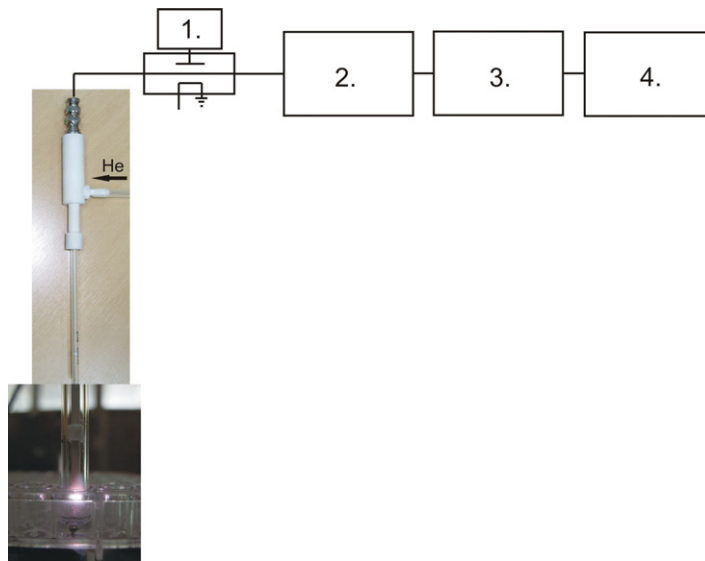
Figure 1. The experimental setup used in treatments: 1, oscilloscope connected to current and voltage derivative probes; 2, matching network; 3, amplifier; 4, signal generator.

2. Experimental setup

2.1. Setup

Non-equilibrium plasma generated by a plasma needle was used for the treatment of bacteria and cells. In this research, standard strains of two bacterial species were used: *S. aureus* (ATCC 25923) and *E. coli* (ATCC 25922). These species were used because of their regular use in protocols for the testing of antibiotics and disinfecting agents. Also, these species of bacteria are frequently found in later stages of many oro-pharyngeal infections. The other cells used in the experiments for cell toxicity were hPB-MSC obtained from a healthy volunteer. The experimental setup used for these treatments is shown in figure 1.

The needle consists of a central electrode that is made of wolfram and is 0.5 mm in diameter, covered almost to the tip by a slightly larger ceramic tube and both placed in a glass tube with a 6 mm inside diameter. The needle body is made of Teflon. Helium is flowing between the ceramic and glass tubes. We used flow rates of 0.5 and 1 slm (standard liters per minute). The central electrode is powered by a 13.56 MHz signal generator (Agilent N9310A) through an amplifier (Barthel RFA-0.1/50–100 B00) and a matching network. The grounded electrode is copper foil placed beneath the plastic plate. Plasma can ignite even without an additional grounding, but in this way we can obtain higher intensities and focus plasma better towards the bacteria suspension. The distance between the surface of the suspension containing bacteria and the tip of the electrode is 3 mm.

In order to measure the power delivered to the plasma, we use derivative probes placed as close as possible to the needle tip. Probe signals are transferred from the oscilloscope to the computer for further manipulation. Firstly, fast Fourier transform is performed followed by calibration of the signals in the frequency domain and subtraction of the displacement current. Finally, converting back to the time domain using inverse fast Fourier transform is carried out.

The difference in signals when the plasma is on and off (no helium flow) carries information about the power delivered to the plasma. Application of probes allows us to gain a direct knowledge of the actual power transmitted to the plasma. Choosing the measured power as a basic parameter provides more direct knowledge of some of the conditions in a plasma and a better control of whether all treatments are done using the same or very similar plasmas every time. This may not be the case when power is measured from the power supply. A suspension containing bacteria was placed inside 96 wells of a microtiter plate and exposed to the plasma generated by the needle. The sample exposure to a severe gas flow can lead to cell injury and contribute to killing [19, 34, 35]. Therefore, to ensure that this was not the case with the gas flows we used during these experiments, we always had control samples that were exposed only to a gas jet without ignition of the plasma.

2.2. Bacteria treatment

Standard strains of bacteria were obtained from American Type Culture Collection (ATCC). These were kept in a deep freeze at a temperature of $-76\,°C$. They were activated by cultivating in dextrose broth (Torlak Institute of Immunology and Virology, Serbia), and incubated in a thermostat for 20 h at $37\,°C$. After incubation, the strains were sub-cultivated in solid nutritional media. *S. aureus* was sub-cultivated on blood agar (Torlak Institute of Immunology and Virology, Serbia) and *E. coli* was sub-cultivated on endo agar (Torlak Institute of Immunology and Virology, Serbia). Sub-cultures on solid nutritional media were incubated under the same conditions (20 h at $37\,°C$).

Further, suspensions with different numbers of bacteria were made. This was done according to 4 McFarland standards whose density corresponds to an approximate cell density of $12 \times 10^8\,CFU\,ml^{-1}$. From this initial suspension, other suspensions were made with cell density 1:10, 1:100 and 1:1000 of the original. The suspensions were made in a microtiter plate with 96 wells. Each well contained 0.18 ml of suspension.

After the treatment of bacterial suspensions with the plasma needle, 0.05 ml of the suspension sample was taken from each well and cultivated on the corresponding solid nutritional media. These solid media were then incubated for 20 h at $37\,°C$, after which the results were analysed. The effect of the plasma needle treatment was graded according to the number of bacterial colonies formed, which are described using arbitrarily defined units 0–5 (0, no growth; 1, sparse growth ($\leqslant 50$ colonies per plate); 2, moderate growth (50–200 per plate); 3, abundant growth (200–500 per plate); 4, very abundant growth (>500 colonies per plate with areas of confluent growth); 5, confluent growth) [36].

The effect of bacteria sterilization using high temperatures is well known [37]. For the purpose of measuring temperature increase during the plasma treatment, we placed a chromel–alumel thermocouple into the suspension 1 mm from the bottom of the well. Measurements were done for both helium flows (0.5 and 1 slm). It is shown that there was no significant increase in sample temperature during the treatment and so the effects of sterilization are only due to the plasma influence.

2.3. MSC treatment

Human MSCs were isolated from mononuclear peripheral blood cells using the methodology described by Kuznetsov *et al* [38], and characterized as plastic adherent cells that display

fibroblastic morphology. After several successive passages, we generated a homogeneous population of MSCs with the capacity to differentiate into osteocytes, adipocytes and myocytes when cultured in the corresponding specific differentiation media [39].

The cells were cultivated in polystyrene flasks at $37\,°C$ in a humidified atmosphere with 5% CO_2. The culture medium was Dulbecco's modified eagle medium (DMEM) with $4.5\,g\,l^{-1}$ glucose, supplemented with 10% fetal bovine serum (FCS), $0.1\,M$ Hepes buffer, $100\,IU\,ml^{-1}$ penicillin and $100\,mg\,ml^{-1}$ streptomycin. About $48\,h$ before treatment with the plasma (the time needed for the cells to reach confluence), cells were seeded in multi-well plates (24- or 48-well plates) at $25\,000$ cells cm^{-2}, and incubated in a culture medium at $37\,°C$ in a humidified atmosphere with 5% CO_2.

The treatment of hPB-MSC with the plasma needle was done in a similar way to the bacteria treatment, and after the treatments, MSC viability and adhesion were tested using the MTT and CV assays, respectively.

2.4. MTT assay

Cell viability was assessed by using the MTT assay. After the treatment, the medium in the wells was replaced by a fresh one and the MTT substrate was added to the final concentration of $0.5\,mg\,ml^{-1}$. The cells were incubated for $3\,h$ at $37\,°C$ in a humidified atmosphere with 5% CO_2. In the mitochondria of living cells, yellow MTT is reduced to water-insoluble purple formazan. The formazan dye was dissolved in absolute isopropanol acidified with $0.1\,N$ HCl and the absorbance of the colored solution was measured by a spectrophotometer at $540\,nm$ [40].

2.5. Adhesion assay

After the treatment, the cells were washed with phosphate-buffered saline (PBS) and fixed with ice-cold methanol for $10\,min$. The adherent cells were then stained by 0.2% solution of CV in PBS for $10\,min$ at room temperature and washed thoroughly with tap water. The stain was dissolved in 33% acetic acid and the absorbance of the colored solution was measured by a spectrophotometer at $540\,nm$ [41]. Photographs of the plasma-treated cells remaining at the bottom of plate wells were also taken, from which areas of cell adhesion and detachment could be determined.

3. Results and discussion

3.1. Plasma treatment of E. coli and S. aureus bacteria

Many factors are responsible for bacterial inactivation. Direct exposure of the bacterial samples to the plasma is always more effective than remote exposure. Even in the case of remote exposure, significant killing can be obtained as well [42]. Another factor that determines the efficiency of the specific treatment is the bacteria sample type [43]. All our samples were prepared as planktonic samples [4, 44]. These are liquid samples with inoculated bacteria, with varying concentrations of bacterial colony-forming units per ml (CFU/ml).

We treated all the samples for three different time periods, three different powers and two different flows of the buffer gas in the needle. For every treatment set, two types of control were used: completely untreated samples and samples treated only by the flow of the buffer gas (He)
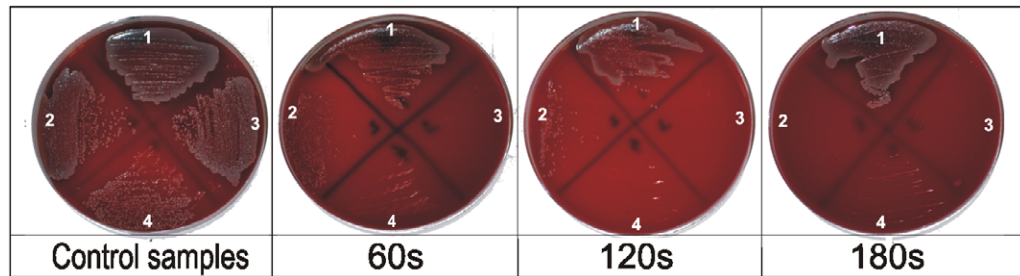
Figure 2. *S. aureus* treated by plasma. The flow rate of He was 0.5 slm and the power was 1.6 W. Four different starting concentrations of bacteria were used: **1**, 12×10^8 CFU ml^{-1}; **2**, 12×10^7 CFU ml^{-1}; **3**, 12×10^6 CFU ml^{-1}; **4**, 12×10^5 CFU ml^{-1}.
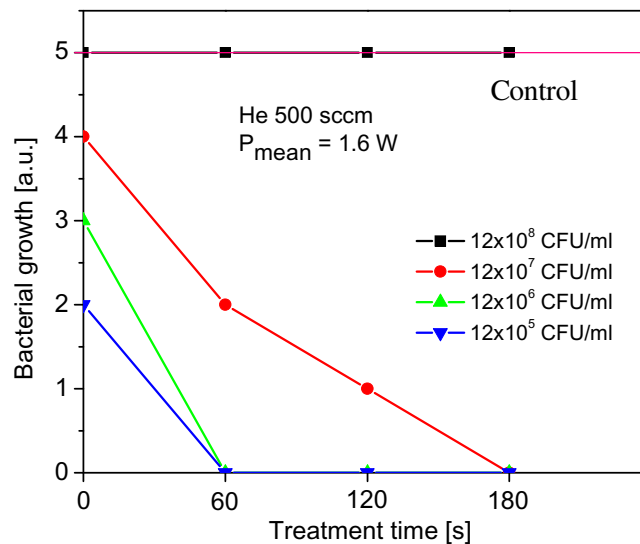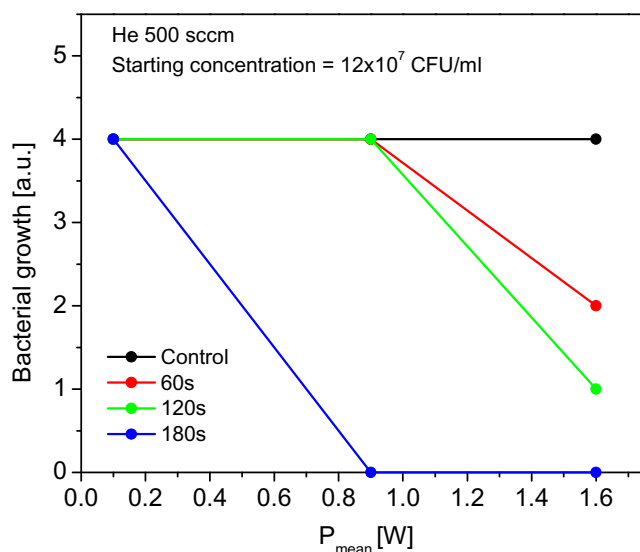

Figure 3. Arbitrarily defined units describing the number of bacterial colonies of *S. aureus* (ATCC 25923) after treatment with plasma. Four samples with different starting concentrations were used; the power was 1.6 W and the He flow rate was 0.5 slm.

but without plasma ignition. In cases where only He flow was present, none of the bacteria were destroyed. After sub-cultivation in solid nutritional media, the bacteria colonies were counted.

In figure 2, photographs of the cultivated bacteria *S. aureus* (ATCC 25923) are shown. The different starting concentrations presented in the figure are marked by numbers (**1**, 12×10^8 CFU ml^{-1}; **2**, 12×10^7 CFU ml^{-1}; **3**, 12×10^6 CFU ml^{-1}; **4**, 12×10^5 CFU ml^{-1}). The flow rate of the buffer gas was 0.5 slm. We can see that for the highest power used almost all bacteria are destroyed except for the highest starting concentration (**1** in figure 2). This pertains only to the conditions that we set here and we did not seek extended treatment to achieve full sterilization for all concentrations.

After the 12 h incubation, bacterial colonies were counted and the results for all four concentrations are shown in figure 3. The effect of the plasma needle treatment was graded according to the number of bacterial colonies formed, which are described using arbitrarily defined units 0–5 (0, no growth; 1, sparse growth; 2, moderate growth; 3, abundant growth;
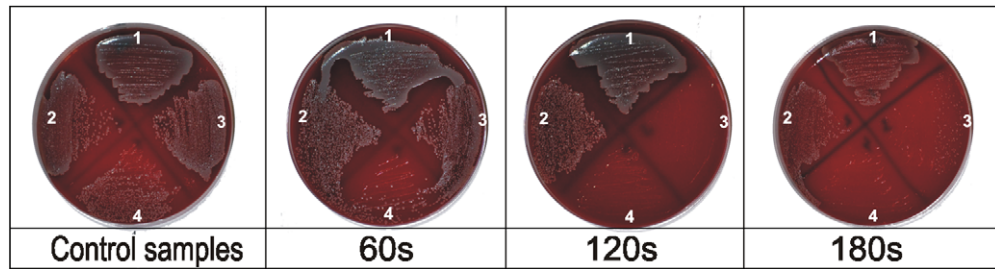
Figure 4. Arbitrarily defined units describing the number of bacterial colonies of *S. aureus* (ATCC 25923) after treatment with the plasma needle as a function of the applied mean power.

4, very abundant growth; 5, confluent growth). The units are chosen to correspond to the growth related to different densities without treatment. In this respect, the results shown in these units may be perceived as a logarithmic representation of the concentration. The results presented in this graph are obtained for the highest power used (1.6 W) and a flow rate of He of 0.5 slm. We can see that with an increase in the treatment time, the number of bacterial colonies formed is significantly reduced. For the longest treatment time except for the highest concentration, practically all the bacteria are killed in the treated sample. For smaller concentrations, even the shortest treatment times were sufficient to destroy the bacteria.

In figure 4, the final concentration of bacteria as a function of applied power in the treatments is shown. Data were obtained for samples with the starting concentration of 12×10^7 CFU ml^{-1}. With an increase in the applied power and with an increase in the time period of treatment, the number of bacterial colonies can be reduced by a factor of 10^4. This means that more than 99% of the bacteria that were contained in the initial sample were killed. By looking at the photographs of the treated samples (see figure 2) visually, we can see that almost no colonies were formed for this set of parameters (highest power/longest treatment time). Most importantly, one can see that for higher initial concentrations, the treatment time proved to be more important than merely the power, which could be related to the fact that our samples were in solution. For example, the 180 s treatment proves to be efficient after a sufficient power level is reached, even at lower powers, whereas higher powers appear to be less efficient for shorter treatment times.

The same set of treatments was performed also for a flow rate of the buffer gas of 1 slm. All other parameters, such as applied power and treatment times, were the same as those for the flow rate of 0.5 slm. Photographs of the cultivated bacteria for a flow rate of 1 slm are shown in figure 5. Again, as in the case of a smaller gas flow, the highest applied power and longest treatment time result in the highest killing of the bacteria. For the two smallest starting concentrations of bacteria, almost no colonies manifest after the treatments.

Figure 5. *S. aureus* treated by plasma. Flow of He was 1 slm and the power was 1.6 W. Four different initial concentrations of bacteria were used: **1**, 12×10^8 CFU ml^{-1}; **2**, 12×10^7 CFU ml^{-1}; **3**, 12×10^6 CFU ml^{-1}; **4**, 12×10^5 CFU ml^{-1}.
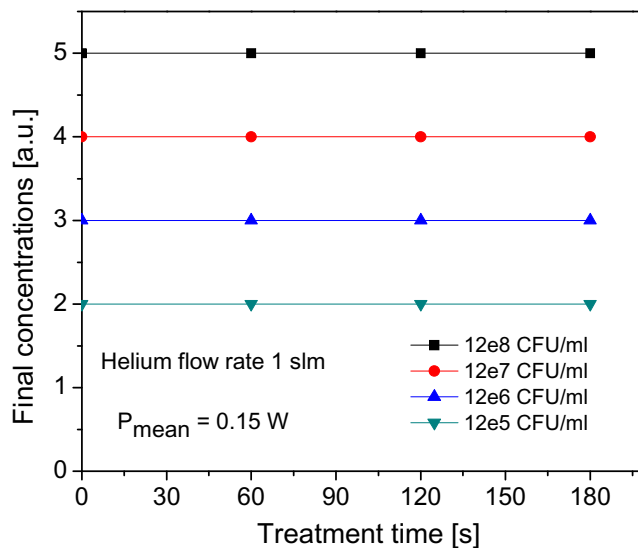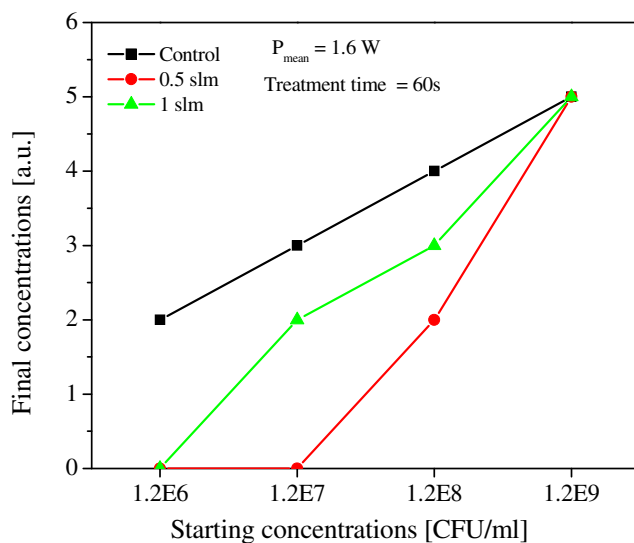

Figure 6. Arbitrarily defined units describing the number of bacterial colonies of *S. aureus* (ATCC 25923) after the treatment as a function of treatment duration for four different starting concentrations.

On the other hand, for the smallest applied power (0.15 W), we could not see any change in bacteria number even after extended treatment times (see figure 6). As in the case of the 0.5 slm flow rate, the lowest power did not reduce the concentration of bacteria in the treated samples.

The flow of the buffer gas plays an important role in the resulting concentrations of bacteria [34]. This is presumably due to different resulting concentrations of reactive particles (radicals) where the flow of the inert buffer gas pushes atmospheric gases further away from the plasma core, and while the densities of UV photons and ions could remain unperturbed, the densities of chemically active radicals would be reduced because of the increasing gas flow. This is, on the other hand, a simplified explanation as operating conditions may be quite different depending on the percentage of atmospheric gases. Thus, an increased flow may result in reduced operating voltage, thereby reducing the efficiency of the production as well.

In figures 7 and 8, the concentration of bacteria after treatment for two different treatment times (60 and 120 s) are shown for several initial densities. The applied power in all cases was

Figure 7. Arbitrarily defined units describing the number of bacterial colonies of *S. aureus* (ATCC 25923) after the plasma treatment for two different gas flows. The treatment time was 60 s and the applied power was 1.6 W.
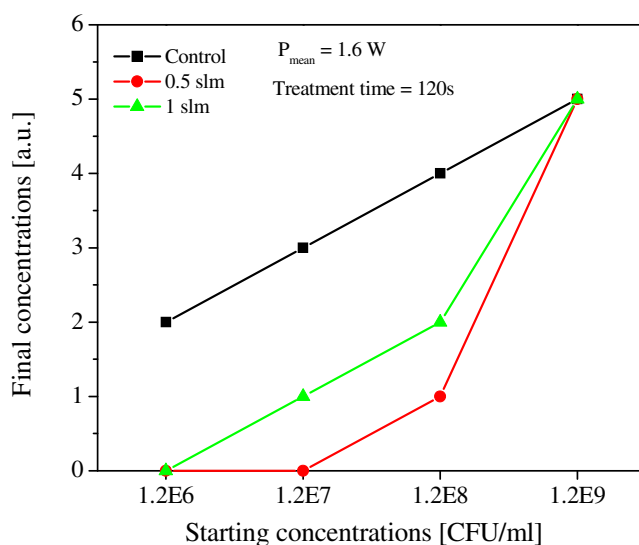
Figure 8. Arbitrarily defined units describing the number of bacterial colonies of *S. aureus* (ATCC 25923) after plasma treatment for two different gas flows. The treatment time was 120 s and the applied power was 1.6 W.

1.6 W. From these figures, it can be concluded that the plasma created when the flow rate of the buffer gas was 0.5 slm was more effective than in the case of the He flow of 1 slm. It is important to note that for both flows of the buffer gas, plasma did not have any effect on the bacteria count in the case of the highest initial concentration. It is perceivable that we could find conditions where this could be achieved, but we decided to operate under conditions where we had a full dynamic range that is accessible to us. Nevertheless, one should be warned that extended treatment times may have to be used for very high densities of bacteria.
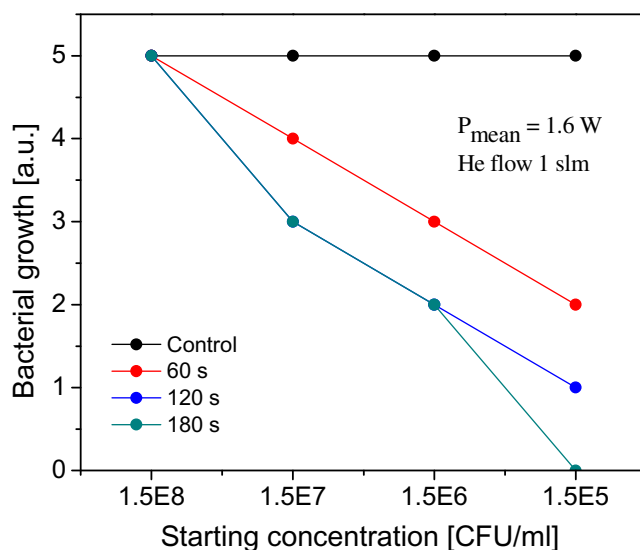
Figure 9. Concentration of the bacteria *E. coli* (ATCC 25922) after plasma treatment for three different treatment times.

We have also done some preliminary treatments of planktonic samples containing *E. coli* (ATCC 25922). *E. coli* is a Gram-negative bacteria, which means that it has an additional outer membrane made of lipopolysaccharides and protein. If mechanical erosion of the bacterial membrane is one of the factors responsible for its inactivation, we can assume that *E. coli* will suffer less damage in the same treatment conditions, as compared to *S. aureus*. Park *et al* [45, 46] showed that damage induced by plasma in the case of *E. coli* consisted of punctured, eroded and morphologically transformed bacteria, while for *S. aureus*, the bacteria were ruptured with their cellular contents released onto the substrate surface. This, according to the authors, demonstrates that a strong etching process is responsible for the observed micro-organism inactivation [45, 46].

In the case of *E. coli*, our starting concentrations were somewhat smaller than in the treatments of *S. aureus*. In figure 9, the concentration of *E. coli* (ATCC 25922) after plasma treatment is shown. Again, even though the highest initial concentration is smaller than in the case of *S. aureus* (1.5×10^8 CFU ml^{-1}), we did not notice any change in bacteria count after the treatments. Better results are obtained for lower initial concentrations. With an increase in the treatment time, the number of bacteria colonies can be reduced even by a factor of 10^4 (almost 99% of the initial concentration).

Some of the agents from the chemical (plasma) point of view that can cause bacterial death or injury include heat, stress, UV radiation, free radicals and charging [43]. Heat-based sterilization methods use either moist heat or dry heat. In the case of moist heat, such as in an autoclave, a temperature of 121 °C at a pressure of 15 psi is used. Dry heat sterilization requires temperatures close to 170 °C and treatment times of about 1 h [37]. For obvious reasons, thermal sterilization cannot be applied to local sterilization of living organisms.

In the case of plasma treatment of the contaminated samples, thermal damage is usually not an important mechanism of injury, considering that most (including plasma needle) discharges at atmospheric pressure operate at room temperature. Since temperature can be one of the possible agents that causes bacterial death or injury, we wanted to check the changes in temperature of
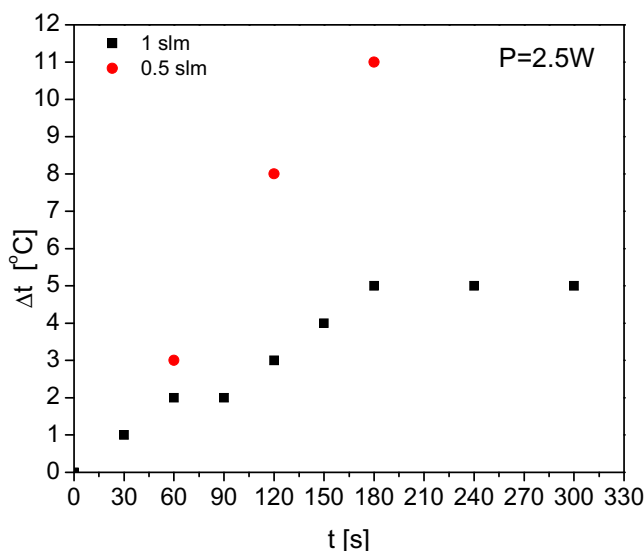
Figure 10. Temperature change of the treated sample as a function of duration of treatment. The flow rates of the buffer gas were 0.5 and 1 slm, and the distance from the tip of the needle to the bacteria-containing liquid was 3 mm.

the sample during treatment. Temperature measurements were done in the same experimental setup used for treatments of bacteria samples. The chromel–alumel thermocouple was used for temperature measurements and it was placed at the bottom of the treated well. The same quantity of saline solution (180 μl) as used in bacteria inactivation was placed in the well.

Temperature measurements were done for several different powers transmitted to the plasma, and the temperature difference for the highest power is shown in figure 10. We can see that for the longest treatment times the increase in temperature is less than 5.5 °C (from the room temperature of 21 °C) in the case of 1 slm gas flow and not more than 10 °C for the plasma with 0.5 slm He flow. In order to show significant effects of the heating, we had to go to a power of 2.5 W, which is considerably higher than the power used in this experiment. In this figure, only the highest power used in temperature measurements is shown because the results for some of the lower powers (<1 W) did not show any increase in the measured temperature, but even the sample temperature decreases. This decrease in the sample temperature can be explained by cooling due to flow of the inert gas. In these cases, heating by the plasma is not enough to raise the temperature of the treated samples and the equilibrium temperature is lower than the starting temperature of the sample (room temperature of 21 °C). Most importantly, saturation was reached after 180 s, indicating that a balance between the released heat and the heat that is taken out by losses has been reached, and further increases in the temperature may be controlled by thermal balance in the broader area that can be controlled.

In all our experiments, power was much lower than the power where we could actually record an increase in the temperature, and treatment times in all our experiments did not exceed 3 min. We can conclude that in our case, heat, as one of the agents that can be responsible for bacteria inactivation or death, does not play an important role.

Another very strong disinfectant is UV radiation [44, 47, 48], but its role in atmospheric plasma sterilization goes from modest [2], [49]–[51] to highly effective [8, 24, 46, 52], depending on the type and concentration of bacteria or spores, the amount and composition
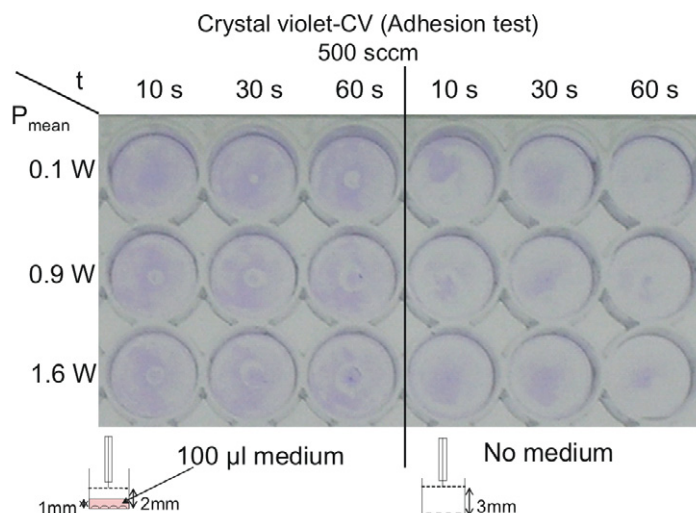
Figure 11. A photograph of a microtiter plate with treated cells after the CV test. Cells are treated using exposure times of 10, 30 and 60 s and mean powers of 0.1, 0.9 and 1.6 W. Wells on the left-hand side contained cells covered by $100 \, \mu$l of medium and those on the right-hand side contained directly exposed cells.

of the medium containing bacteria and the UV radiation power. Besides local heating and UV photons that can be responsible for killing of bacteria, we also have ions created in the discharge, radicals and the effect of charging of the cell membrane.

We have shown that heating of the cells did not exceed the normal conditions. It is very difficult to find a simple mechanism that would allow the effect of radicals and energetic ions to penetrate the liquid. On the other hand, the effect of UV photons would depend strongly on the absorption of UV radiation, and some recent measurements have confirmed that there is a correlation between the effect of plasma and the transparency of the liquid [8]. However, we cannot give a final conclusion on this mechanism in our system without further studies. The negative charging of the cell wall by plasma electrons was considered by Laroussi *et al* [43, 53]. In certain conditions, mechanical stress induced by charging can be sufficient to cause rupture in G− (*E. coli*) but not in G+ (*S. aureus*) bacteria. Furthermore, inflicting mechanical damage caused by charging is not the only way to destroy cells. If the surface charge equilibrium [54]–[58] is disturbed, cells can be killed without actually tearing them apart. Charging of the bacteria cell wall by electrons in our case is highly improbable because of the fact that bacteria are diluted in saline solution that cannot be penetrated by charged particles from plasmas, and also any charge accumulation is conducted away.

3.2. Plasma treatment of human peripheral blood-derived MSC

Along with the plasma treatment of bacteria, we have also performed plasma treatment of hPB-MSC to test the possible harmful effect of plasmas on normal, living cells. Plasma parameters in these experiments were the same as those when bacteria samples were treated. After the cell treatment, two types of tests were performed: the MTT assay for cell viability, and the CV staining assay as a measure of the cells' adhesion.

In figure 11, a photograph of the adhesion test after the plasma treatment is shown. On the left-hand side (3 × 3) are wells in which the cells were covered with $100 \, \mu$l of DMEM during the
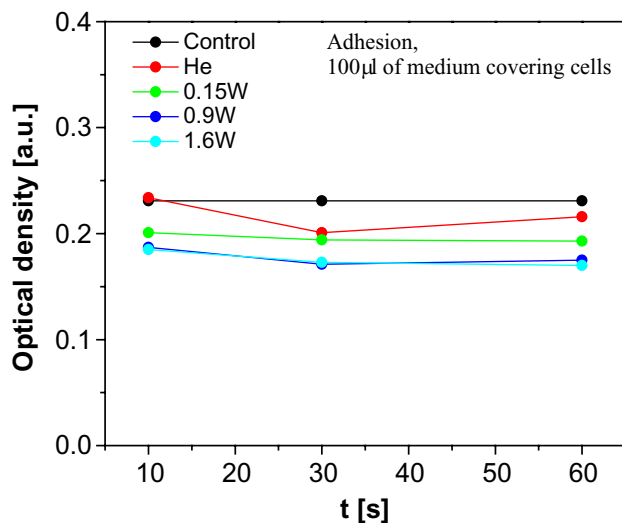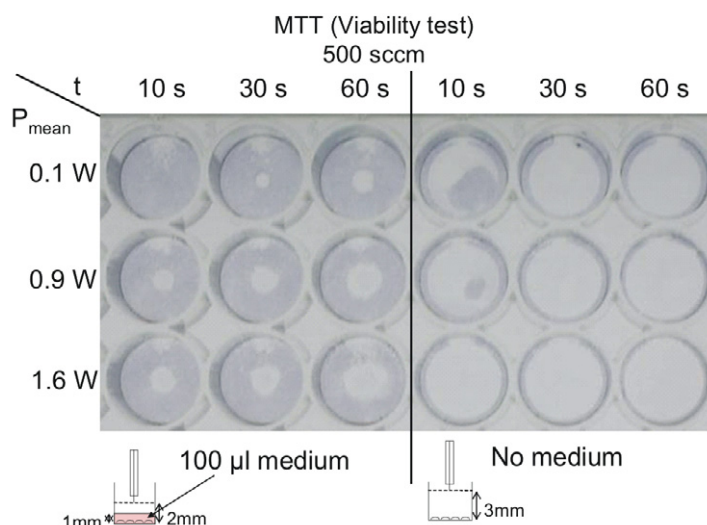
Figure 12. Optical densities of treated cells covered by $100\,\mu$l of medium obtained by a spectrophotometer at 540 nm (CV tests).
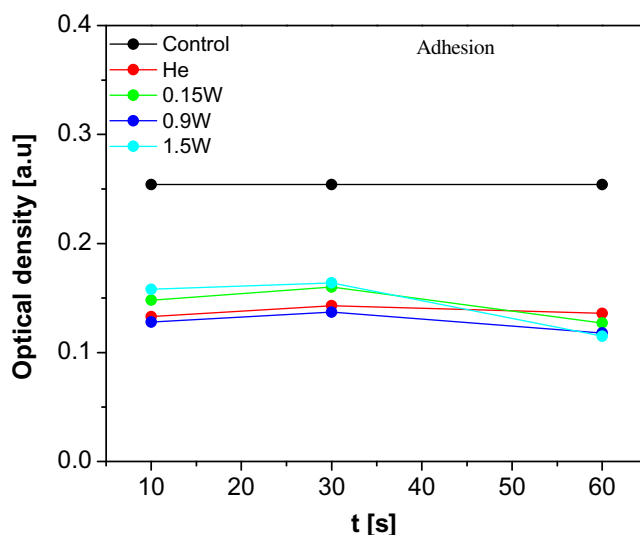

Figure 13. Optical densities of directly treated cells obtained by a spectrophotometer at 540 nm (CV tests).

treatment. As shown in both figures 11 and 12, it can be seen that during the plasma treatment, only a small number of cells were detached. With an increase in the applied power, only a small change is noticeable, while there is almost no change in the number of attached cells with a variation in the treatment time. In all cases, He control was performed where there was an He flow (in this case 0.5 slm), but the plasma was not ignited, and we noticed a certain amount of cells that were detached even in this case when flow was present in the absence of plasma.

On the other hand, in the well in which there was no medium covering treated cells (3 × 3—right-hand side of figure 11), detachment of the cells during the treatment was quite high and He flow played the most important role in this case (see figure 13). Here, one cannot distinguish between the effect of plasma and the effect of He flow.

Figure 14. A photograph of a microtiter plate containing treated cells after the MTT (viability) test. Cells are treated using exposure times of 10, 30 and 60 s and mean powers of 0.1, 0.9 and 1.6 W. The left-hand side wells contained cells covered by $100\,\mu$l of medium, while the right-hand side wells contained directly exposed cells.
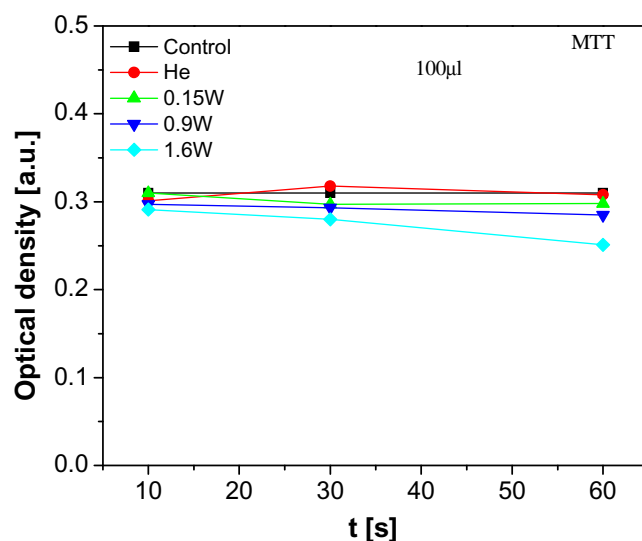
Figure 15. Optical densities of treated cells covered by $100\,\mu$l of medium obtained by a spectrophotometer at 540 nm (MTT tests).

The results of the viability tests performed are presented in figure 14, showing the photograph of the wells after the plasma treatment and MTT test coloring. Again, on the left-hand side of the photograph (3×3) are wells in which the cells were covered with $100\,\mu$l of medium during the treatment. In this case, we could notice that more cells were dead with an increase in the applied power and treatment time. Control with only He flow shows no changes at all (see figure 15).
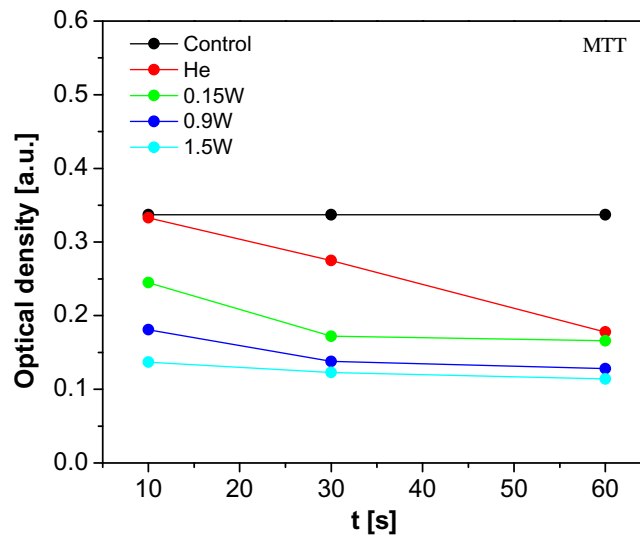
Figure 16. Optical densities of directly treated cells obtained by a spectrophotometer at 540 nm (MTT tests).
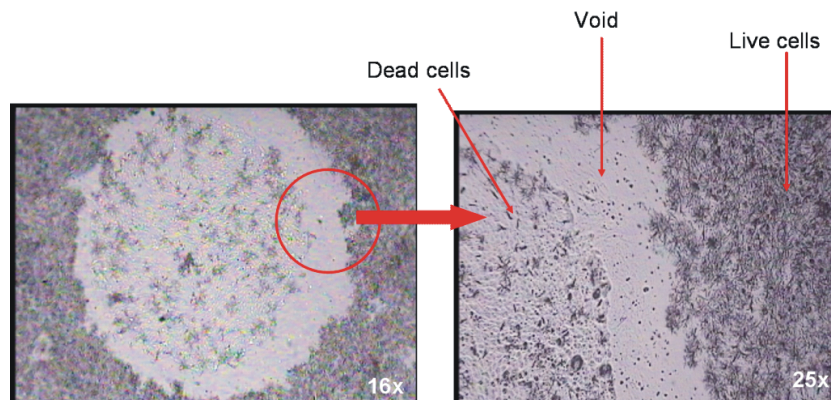
Figure 17. Photographs of the representative well containing hPB-MSC covered by 100 μl of medium after plasma treatment and the MTT test.

The right-hand side of the photograph (3×3 wells; see figure 14) shows the wells in which there was no medium covering the cells during treatment. Again, control of He flow was done, and with an increase in treatment time more cells were destroyed, even if no plasma was ignited (see figure 16). By increasing the power and treatment time, drastic changes in viability percentage of the cells were observed.

The photograph of the plasma-treated hPB-MSC after the MTT test, shown in figure 17, points to the 'track' of the plasma left on the mesenchymal cell sample in the plate wells. It can clearly be seen that there are three separate regions with cells exhibiting different responsiveness. The first region is at the center of the well, where the cells were placed directly under the tip of the needle during treatment. All the cells in this area were destroyed, although some of their debris still adheres to the surface. At the edge of this region, there is an annular area, a gap without any cell present. Living cells can be observed only outside this area towards the wall of the well.

Both the viability and the adhesion tests demonstrated that the effect of plasma+He treatment was somewhat milder when the cells during the treatment were covered with medium as compared to the case when the cells were without medium, i.e. in direct contact with the plasma, which makes its effect stronger and more efficient in terms of cell removal. Under these conditions, however, it was shown that the gas flow played an even more important role, which is not the case when liquid medium is present over the cell samples. This result indicates that the plasma parameters should be adjusted according to the treatment desired. Namely, because in the human body the cells are within a liquid microenvironment, the plasma parameters when plasma is applied for pathological cell or tissue removal or for wound healing have to be somewhat different from those in the case of direct contact with the tissue, for example skin peeling. Further work on optimization of the plasma parameters should include experiments on MSCs differentiated toward specific tissues, along with defining mechanisms underlying the cell responses.

4. Conclusion

This work continues our earlier work on plasma-induced sterilization at low pressures [59], plasma treatment of seeds at low and atmospheric pressures [60, 61] and the effects of a plasma needle on the calli *Fritillaria imperialis* (family Lilliaceae) [62]. The main goal of this paper was to study the effects of plasma treatment on two cell systems. The treatment of *E. coli* and *S. aureus* bacteria was performed in order to study the deactivation of harmful bacteria, while the MSCs were a model system to predict the degree of possible damage to cell responses.

In the treatment of *S. aureus* (ATCC 25923), we see a very good effect of plasma, practically no effect of the gas flow and sterilization of the order of five orders of magnitude in some cases. At very low powers there is no effect, but at higher powers the effect increases dramatically with treatment time. Finally, very little effect was found when the initial densities were relatively high for the choice of powers that were selected in this study. We have also verified that in addition to gas flow, the heating was low and played no role in the sterilization of samples. Similar but somewhat less impressive results were found for *E. coli* (ATCC 25922). This reduction in efficiency may be expected for Gram-negative bacteria but still the results in both cases support very efficient sterilization and give a promise of applications in sterilization of living tissues and cavities. For very high initial densities in both cases, the effect of plasma was not observed, requiring higher powers, or perhaps more optimal choice of flow and proximity. Independent of our work, a study with *E. coli* and *S. aureus* in planktonic samples was recently published by Joshi *et al* [9], reaching similar conclusions.

For hPB-MSC, measurements were made when the cells were covered with medium as well as when the cells were without medium. In the case of direct cell exposure, without medium cover, although the effect of the plasma was observable, its impact was diminished in the context of the desiccation caused by the gas flow. When the cells were covered by medium, gas flow did not affect the results, and the effect of the plasma was more obvious. The effect differed in three regions. The central circle of the plasma-treated area consisted of dead cells; the annular region with no cell debris, perhaps defined by the anatomy of the plasma in a plasma needle, due to the combined effect of gas flow, mixing with the atmosphere and the central core of plasma with energetic and reactive particles; and finally, the outside region consisted of the viable cells. Since the region with dead cells could be controlled by defined plasma parameters (power, duration of treatment), their optimization can lead to refined cell removal.

Comparisons of the effects of plasmas on bacteria and on human cells may be justified by the search for selectivity that may be necessary for the treatment of live tissues. As far as plasma goes, some further optimization may be achieved for localized accurate treatment of cells or sterilization. Further work needs to be done to achieve optimal operation and uniformity over large areas. With a good knowledge of the power deposited into the plasma, and control of the radicals that are produced, together with spatial emission profiles indicating changes to the regime of operation, sufficient control of the reproducibility of plasma needle operation is achieved. Other sources may be sought for more refined interaction with living cells.

Acknowledgments

References

[1] Fridman A and Kennedy L A 2004 *Plasma Physics and Engineering* (New York: Taylor and Francis)
[2] Iza F, Kim G J, Lee S M, Lee J K, Walsh J L, Zhang Y T and Kong M G 2008 Microplasmas: sources, particle kinetics, and biomedical applications *Plasma Process. Polym.* **5** 322–44
[3] Stoffels E, Flikweert A J, Stoffels W W and Kroesen G M W 2002 Plasma needle: a nondestructive atmospheric plasma source for fine surface treatment of (bio)materials *Plasma Sources Sci. Technol.* **11** 383–8
[4] Sladek R E J, Stoffels E, Walraven R, Tielbeek P J A and Koolhoven R A 2004 Plasma treatment of dental cavities: a feasibility study *IEEE Trans. Plasma Sci.* **32** 1540–3
[5] Moisan M, Barbea J, Crevier M C, Pelletie J, Philip N and Saoudi B 2002 Plasma sterilization. Methods and mechanisms *Pure Appl. Chem.* **74** 349–58
[6] Kutasi K, Pintassilgo C D and Loureiro J 2009 An overview of modelling of low-pressure post-discharge systems used for plasma sterilization *J. Phys.: Conf. Ser.* **162** 012008
[7] Dobrynin D, Fridman G, Friedman G and Fridman A 2009 Physical and biological mechanisms of direct interaction with living tissue *New J. Phys.* **11** 115020–46
[8] Nosenko T, Shimizu T and Morfill G E 2009 Designing plasmas for chronic wound disinfection *New J. Phys.* **11** 115013–32
[9] Joshi S G, Paff M, Friedman G, Fridman G, Fridman A and Brooks A D 2010 *Am. J. Inf. Control* **38** 293–301
[10] Fridman G, Friedman G, Gutsol A, Shekhter A B, Vasilets V N and Fridman A 2008 Applied plasma medicine *Plasma Process. Polym.* **5** 503–33
[11] Fridman G, Shereshevsky A, Jost M M, Brooks A D, Fridman A, Gutsol A, Vasilets V and Friedman G 2007 Floating electrode dielectric barrier discharge plasma in air promoting apoptotic behavior in melanoma skin cancer cell lines *Plasma Chem. Plasma Process.* **27** 163–76
[12] Lee M H, Park B J, Jin S C, Kim D, Han I, Kim J, Hyun S O, Chung K-H and Park J-C 2009 Removal and sterilization of biofilms and planktonic bacteria by microwave-induced argon plasma at atmospheric pressure *New J. Phys.* **11** 115022–33
[13] Morfill G E, Kong M G and Zimmermann J L 2009 Focus on plasma medicine *New J. Phys.* **11** 115011–9
[14] Kim G C, Kim G J, Park S R, Jeon S M, Seo H J, Iza F and Lee J K 2009 Air plasma coupled with antibody-conjugated nanoparticles: a new weapon against cancer *J. Phys. D: Appl. Phys.* **42** 032005–10

[15] Kim G J, Kim W, Kim K T and Lee J K 2010 DNA damage and mitochondria dysfunction in cell apoptosis induced by non-thermal air plasma *Appl. Phys. Lett.* **96** 021502–5

[16] Lazović S, Puač N, Malović G, Đorđić A and Petrović Z Lj 2008 Diagnostic of plasma needle properties by using mass spectrometry *Chem. Listy* **102** s1383–7

[17] Malović G, Puač N, Lazović S and Petrović Z Lj 2010 Mass analysis of an atmospheric pressure plasma needle discharge *Plasma Sources Sci. Technol.* **19** 034014

[18] Puač N 2008 Development, diagnostic and applications of radio-frequency plasma reactor *J. Phys.: Conf. Ser.* **133** 012007

[19] Goree J, Liu B, Drake D and Stoffels E 2006 Killing of *S. mutans* bacteria using a plasma needle at atmospheric pressure *IEEE Trans. Plasma Sci.* **34** 1317–24

[20] Sladek R E J and Stoffels E 2005 Deactivation of *Escherichia coli* by the plasma needle *J. Phys. D: Appl. Phys.* **38** 1716–21

[21] Kelly-Wintenberg K, Hodge A, Montie T C, Deleanu L, Sherman D, Reece Roth J, Tsai P and Wadsworth L 1999 Use of a one atmosphere uniform glow discharge plasma to kill a broad spectrum of microorganisms *J. Vac. Sci. Technol.* A **17** 1539–44

[22] Heinlin J, Isbary G, Stolz W, Morfill G, Landthaler M, Shimizu T, Steffes B, Nosenko T, Zimmermann J L and Karrer S 2010 Plasma applications in medicine with a special focus on dermatology *J. Eur. Acad. Dermatol. Venereol.* published online: 17 May 2010, DOI: 10.1111/j.1468-3083.2010.03702.x

[23] Morfill G E, Shimizu T, Steffes B and Schmidt H-U 2009 Nosocomial infections—a new approach towards preventive medicine using plasmas *New J. Phys.* **11** 115019–29

[24] Sato T, Miyahara T, Doi A, Ochiai S, Urayama T and Nakatani T 2006 Sterilization mechanism for *Escherichia coli* by plasma flow at atmospheric pressure *Appl. Phys. Lett.* **89** 073902

[25] Laroussi M 2002 Nonthermal decontamination of biological media by atmospheric-pressure plasmas: review, analysis, and prospects *IEEE Trans. Plasma Sci.* **30** 1409–15

[26] Yu Q S, Huang C, Hsieh F H, Huff H and Duan Y 2006 Sterilization effects of atmospheric cold plasma brush *Appl. Phys. Lett.* **88** 013903

[27] Kieft I E, Kurdi M and Stoffels E 2006 Reattachment and apoptosis after plasma-needle treatment of cultured cells *IEEE Trans. Plasma Sci.* **34** 1331–6

[28] Stoffels E, Kieft I E, Sladek R E J, van den Bedem L J M, van der Laan E P and Steinbuch M 2006 Plasma needle for *in vivo* medical treatment: recent developments and perspectives *Plasma Sources Sci. Technol.* **15** S169–80

[29] Kieft I E, Dvinskikh N A, Broers J L V, Slaaf D W and Stoffels E 2004 *Proc. SPIE* **5483** 247

[30] Kong M 2009 personal communication

[31] Chamberlain G, Fox J, Ashton B and Middleton J 2007 Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing *Stem Cells* **25** 2739–49

[32] Chang J K, Li C J, Wu S C, Yeh C H, Chen C H, Fu Y C, Wang G J and Ho M L 2007 Effects of anti-inflammatory drugs on proliferation, cytotoxicity and osteogenesis in bone marrow mesenchymal stem cells *Biochem. Pharmacol.* **74** 1371–82

[33] Dai Z Q, Wang R, Ling S K, Wan Y M and Li Y H 2007 Simulated microgravity inhibits the proliferation and osteogenesis of rat bone marrow mesenchymal stem cells *Cell Prolif.* **40** 671–84

[34] Goree J, Liu B and Drake D 2006 Gas flow dependence for plasma-needle disinfection of *S. mutans* bacteria *J. Phys. D: Appl. Phys.* **39** 3479–86

[35] Laroussi M and Akan T 2007 Arc-free atmospheric pressure cold plasma jets: a review *Plasma Process. Polym.* **4** 777–88

[36] Miller P H, Wiggs L S and Miller J M 1995 Evaluation of anaerogen system for growth of anaerobic bacteria *J. Clin. Microbiol.* **33** 2388–91

[37] Block S S 1992 Sterilization *Encyclopedia of Microbiology* vol 4 ed J Lederberg (San Diego, CA: Academic) p 87

[38] Kuznetsov S A, Mankani M H, Gronthos S, Satomura K, Bianco P and Robey P G 2001 Circulating skeletal stem cells *J. Cell Biol.* **153** 1133–40

[39] Kocic J unpublished results

[40] Mosmann T 1983 Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assay *J. Immunol Methods* **65** 55–63

[41] Oez S, Welte K, Platzer E and Kalden J R 1990 A simple assay for quantifying the inducible adherence of neutrophyls *Immunobiology* **180** 308–15

[42] Stolz W, Georgi M, Schmidt H U, Ramrath K, Pompl R, Shimizu T, Steffes B, Bunk W, Peters B, Jamitzky F and Morfill G 2007 Low-temperature argon plasma for sterilization of chronic wounds: from bench to bedside *Proc. 1st Int. Conf. on Plasma Med. (Corpus Christi, TX, 15–18 October 2007)* p. 15

[43] Stoffels E, Sakiyama Y and Graves D B 2008 Cold atmospheric plasma: charged species and their interactions with cells and tissues *IEEE Trans. Plasma Sci.* **36** 1441

[44] Kamgang J O, Briandet R, Herry J M, Brisset J L and Naitali M 2007 Destruction of planktonic, adherent and biofilm cells of *Staphylococcus epidermidis* using a gliding discharge in humid air *J. Appl. Microbiol.* **103** 621–8

[45] Park B J, Lee D H, Park J C, Lee I S, Lee K Y, Hyun S O, Chun M S and Chung K H 2003 Sterilization using a microwave-induced argon plasma system at atmospheric pressure *Phys. Plasmas* **10** 4539–44

[46] Lee K Y, Joo Park B, Hee Lee D, Lee I S, Hyun O S, Chung K H and Park J C 2005 Sterilization of *Escherichia coli* and MRSA using microwave-induced argon plasma at atmospheric pressure *Surf. Coat. Technol.* **193** 35–8

[47] Sosnin E A, Stoffels E, Erofeev M V, Kieft I E and Kunts S E 2004 The effects of UV irradiation and gas plasma treatment on living mammalian cells and bacteria: a comparative approach *IEEE Trans. Plasma Sci.* **32** 1544–50

[48] Moisan M, Barbeau J, Moreau S, Pelletier J, Tabrizian M and Yahia L'H 2001 Low-temperature sterilization using gas plasmas: a review of the experiments and an analysis of the inactivation mechanisms *Int. J. Pharm.* **226** 1–21

[49] Laroussi M and Leipold F 2004 Evaluation of the roles of reactive species, heat, and UV radiation in the inactivation of bacterial cells by air plasmas at atmospheric pressure *Int. J. Mass Spectrom.* **233** 81–6

[50] Laroussi M 2005 Low temperature plasma-based sterilization: overview and state-of-the-art *Plasma Process. Polym.* **2** 391–400

[51] Gaunt L F, Beggs C B and Georghiou G E 2006 Bactericidal action of the reactive species produced by gas-discharge nonthermal plasma at atmospheric pressure: a review *IEEE Trans. Plasma Sci.* **34** 1257–69

[52] Moisan M, Saoudi B, Crevier M C, Philip N, Fafard E, Barbeau J and Pelletier J 2003 Recent development in the application of microwave discharges to the sterilization of medical devices *5th Int. Workshop on Microwave Discharges (Greifswald, Germany)* pp 210–21

[53] Laroussi M, Mendis D A and Rosenberg M 2003 Plasma interaction with microbes *New J. Phys.* **5** 41

[54] Bayat O, Arslan V, Bayat B and Poole C 2004 Application of a flocculation–ultrafiltration process for bacteria (*Desulfovibrio desulfuricans*) removal from industrial plant process water *Biochem. Eng. J.* **18** 105–10

[55] Wasserman E and Felmy A R 1998 Computation of the electrical double layer properties of semipermeable membranes in multicomponent electrolytes *Appl. Environ. Microbiol.* **64** 2295–300

[56] West R J, Stephens G M and Cilliers J J 1998 Zeta potential of silver absorbing *Thiobacillus ferrooxidans Minerals Eng.* **11** 189–94

[57] Campanhã M T, Mamizuka E M and Carmona-Ribeiro A M 1999 Interactions between cationic liposomes and bacteria: the physical chemistry of the bactericidal action *J. Lipid Res.* **40** 1495–500

[58] Kuyyakanond T and Quesnel L B 1992 The mechanism of action of chlorhexidine *FEMS Microbiol. Lett.* **100** 211–5

[59] Manola S, Petrović Z Lj and Jankov R M 1993 Application of microwave discharges for sterilization of medical instruments *16th SPIG XVI Summer School and Int. Symp. on Physics of Ionized Gases* (*Belgrade*) ed M Milosavljević p 285

[60] Puač N, Petrović Z Lj, Živković S, Giba Z, Grubišić D and Đorđević A R 2005 Low temperature treatment of dry Empress-tree seeds *Plasma Processes and Polymers* ed R d'Agostino, P Favia, C Oehr and M R Wertheimer (New York: Wiley) pp 193–203

[61] Živković S, Puač N, Giba Z, Grubišić D and Petrović Z Lj 2004 The stimulatory effect of non-equilibrium (low temperature) air plasma pretreatment on light-induced germination of *Paulownia tomentosa* seeds *Seed Sci. Technol.* **32** 693–701

[62] Puač N, Petrović Z Lj, Malović G, Đorđević A, Živković S, Giba Z and Grubišić D 2006 Measurements of voltage–current characteristics of a plasma needle and its effect on plant cells *J. Phys. D: Appl. Phys.* **39** 3514–9