

Article

Inhibitory Effect of Cold Atmospheric Plasma on Chronic Wound-Related Multispecies Biofilms

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Abstract: The presence of microbial biofilms in the wounds affects negatively the healing process and can contribute to therapeutic failures. This study aimed to establish the effective parameters of cold atmospheric plasma (CAP) against wound-related multispecies and monospecies biofilms, and to evaluate the cytotoxicity and genotoxicity of the protocol. Monospecies and multispecies biofilms were formed by methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas aeruginosa* and *Enterococcus faecalis*. The monospecies biofilms were grown in 96 wells plates and multispecies biofilm were formed on collagen membranes. The biofilms were exposed to helium CAP for 1, 3, 5 and 7 min. In monospecies biofilms, the inhibitory effect was detected after 1 min of exposure for *E. faecalis* and after 3 min for MRSA. A reduction in *P. aeruginosa* biofilm's viability was detected after 7 min of exposure. For the multispecies biofilms, the reduction in the overall viability was detected after 5 min of exposure to CAP. Additionally, cytotoxicity and genotoxicity were evaluated by MTT assay and static cytometry, respectively. CAP showed low cytotoxicity and no genotoxicity to mouse fibroblastic cell line (3T3). It could be concluded that He-CAP showed inhibitory effect on wound-related multispecies biofilms, with low cytotoxicity and genotoxicity to mammalian cells. These findings point out the potential application of CAP in wound care.

Keywords: wounds; biofilm; plasma jet

1. Introduction

Chronic wounds, such as venous ulcers, diabetic foot ulcers, and pressure ulcers, are considered one of the most challenging problems in the medical area and affect millions of patients worldwide [1,2]. The prevalence of chronic wounds is estimated in 2.21 per 1000 in the population [3] and these rates tend to rise due to the ageing population and increasing prevalence of obesity and associated conditions, such as diabetes and cardiovascular diseases [4]. This condition has significant economic impact on health systems [2,5], with 2 to 3% of the budget in health applied in the treatment of chronic wounds in developed countries [1].

Microbial biofilms significantly affect wound healing and can contribute to therapeutic failures [6]. For these reasons, they have emerged as an important topic of discussion in the treatment of wounds [7]. The infections of wounds are usually polymicrobial, as their microenvironment is favorable for microbial growth [8,9]. Despite this, previous evidence showed that species diversity in infected wounds is relatively low. It has been reported that *Staphylococcus aureus*, *Enterococcus faecalis* and *Pseudomonas aeruginosa* are the most prevalent species [4,10], with occurrence of 93.5%, 71.7% and 52.2%, respectively [11]. Other bacterial species, such as *Proteus mirabilis*, *Klebsiella pneumoniae* and *Escherichia coli* were also isolated from wounds [12,13]. The increasing rates of antimicrobial resistance also contributes to the unfavorable scenario [14,15].

Thus, the control of the microbial biofilms is considered critical to the success of wound healing [7,16]. The conventional therapeutic alternatives are focused on the reduction or eradication of biofilms by using debridement techniques and treatments with negative pressure or ultrasound [17,18]. Other methods include the use of wound dressing impregnated with antimicrobial substances or compounds, such as gentian violet and methylene blue [15], honey [19], *Aloe vera* [20], nitric oxide releasing polymer [21], polyhexamethylenebiguanides [22] and ethylenediamine tetra acetic acid (EDTA) [23]. However, there is still need for alternatives to wound care, in particular, focusing on biofilm control [17,21,24].

Cold atmospheric plasma (CAP) has emerged as an attractive alternative approach to the biofilm control and is considered a rapid, environmental-friendly, energy saving, and versatile technology [25,26]. Plasma is described as the fourth state of matter and is usually generated from a gaseous precursor in which a high electric field is applied, producing an electric discharge [27]. Plasma is a complex mixture composed of ions, reactive radicals, neutral particles, molecules and electrons, as well as excited species and energetic photons [28,29]. These plasma components can damage cell structures, such as lipids, protein and DNA, and increase the levels of intracellular reactive species [30]. Thus, the biological activity of plasma is a product of synergetic action of several factors, such as neutral reactive species (oxygen reactive species—ROS, reactive nitrogen species—RNS) and UV radiation [31]. The anti-inflammatory and tissue repair inducing [32,33] effects have been considered highly promising for the treatment of several diseases.

The efficiency of CAP against the bacterial biofilms has been reported in the literature [34]. CAP was able to reduce the viability of monospecies biofilms of methicillin resistant *S. aureus* (MRSA) [35], *P. aeruginosa* [36] and *E. faecalis* [37]. Monospecies biofilms formed by *E. faecalis* [38,39] or *Escherichia coli* [40] were also inhibited by CAP. One of the main advantages of CAP when compared to conventional antibiotics is that the development of treatment resistance was not detected so far. Repeated exposure to CAP did not induce resistance in *S. aureus* [41]. The hypothesis is that as CAP does not have specific target structures [35], the chance of resistance development with plasma is lower since several targets are activated and not a specific one [42]. Besides the antimicrobial activity, anti-inflammatory and tissue repair inducing effects (stimulating cellular proliferation and migration and the pro-angiogenic effects) of CAP have been also reported [14,32,43,44].

The biological effects of CAP lead to the hypothesis that this technology can be useful for the treatment of infected wounds. However, to the best of our knowledge, little is known on the effects of CAP on wound-related multispecies biofilms. Thus, the present work aimed to study the effect of He-CAP on wound-related multispecies biofilms and confirm the safety of the protocol.

2. Materials and Methods

2.1. Plasma Jet Device

The plasma jet device used in this study consists of a plasma reactor with dielectric barrier discharge (DBD) configuration. The plasma generated in this primary DBD reactor is transported through a 1.0-m-long, flexible polyurethane tube (Kangaroo TM Nasogastric Feeding Tube, 10 Fr/Ch), connected to the reactor exit. A thin metal wire is installed inside the plastic tube acting as floating electrode. The wire penetrates few millimeters inside

the primary discharge chamber, crosses the whole tube length and terminates about 2 mm before the plastic tube exit. When high voltage is applied to the primary DBD reactor, a remote plasma plume is ignited at the downstream end of the plastic tube. This device was already described in previous works [45–48]. In the present study, the reactor was connected to a Minipuls4 AC power supply (GBS Elektronik GmbH, Radeberg, Germany) and an amplitude modulated voltage signal (12 kV amplitude) with voltage duty cycle of 22% was employed. The system was fed with helium (99.5% purity) at a flow rate of 2.0 slm. The distance between the plastic tube tip and the surface to be treated was set at 1.5 cm.

The characteristic optical emission spectrum of this plasma jet was previously reported by Kostov et al. and Nishime et al., [45–48]. Briefly, weak emission lines of helium were detected with nitrogen emission bands being predominant in the spectrum, showing that excited nitrogen (N_2 -second positive system) and ionized nitrogen species (N_2^+ -first negative system) were produced in the plasma plume. Peaks associated to atomic oxygen (777 nm and 844 nm) and the OH band at 308 nm were also present indicating the production of ROS.

2.2. Bacterial Strains and Growth Conditions

The bacterial strains used in this study were: methicillin resistant *S. aureus* (MRSA) (ATCC 33591), *P. aeruginosa* (ATCC 27853) and *E. faecalis* (ATCC 29212). Strains were grown in tryptic soy agar for 24 h at 37 °C, under aerobiosis. After this period, standardized suspensions containing 10^8 cells/mL in sterile saline solution (NaCl 0.9%) were prepared with the aid of a spectrophotometer (*P. aeruginosa*: $\lambda = 600$, OD 0.115; *E. faecalis*: $\lambda = 760$, OD 1.278 and MRSA: $\lambda = 600$, OD 0.462). Then, these suspensions were diluted in TS broth obtaining the final concentration of 10^6 cells/mL.

2.3. Effect of CAP on Monospecies Biofilms

Monospecies bacterial biofilms of MRSA (ATCC 33591), *P. aeruginosa* (ATCC 27853) and *E. faecalis* (ATCC 29212) were grown in 96 wells plates, as these experiments aimed to standardize the ideal parameters of plasma device in a cost-effective manner. Standardized suspension (10^6 cells/mL) of each strain in Tryptic soy broth (200 μ L) was added to each well. Plates were incubated for 90 min at 37 °C and 80 rpm for initial adherence. After this period, the culture medium was removed, and the wells were washed twice with physiologic solution (NaCl 0.9%). Then, 200 μ L of fresh Tryptic soy broth medium were added to each well and the plates were incubated for 48 h. Subsequently, the culture medium was removed, and the biofilms were washed twice with physiologic solution (NaCl 0.9%). Biofilms were exposed to CAP for 1, 3, 5 and 7 min. A non-exposed treated group was included for comparative purposes. The number of viable cells was determined after serial dilution in physiologic solution and plating on brain heart infusion agar. The results were expressed as colony forming units per mL (CFU/mL). Three independent experiments were performed in triplicate.

2.4. Effect of CAP on Multispecies Biofilms

Multispecies biofilms were formed by MRSA (ATCC 33591), *P. aeruginosa* (ATCC 27853) and *E. faecalis* (ATCC 29212) on collagen membranes (Geistlich Bio-Gide®), according to the methodology described by [49,50]. First, standardized suspensions containing 10^6 cells/mL in physiologic solution (NaCl 0.9%) of each microorganism were prepared spectrophotometrically, using the same parameters described before. The collagen membranes were positioned on the surface of tryptic soy agar supplemented by fetal bovine serum solution (50% fetal bovine serum and 50% NaCl 0.9% solution). The smooth layer of the collagen membrane was considered for the growth of multispecies biofilm. Then, aliquots of 10 μ L of each bacterial suspension were mixed up in a microtube and the total volume was transferred to the membrane's surface. Plates were incubated at 37 °C for 24 h. Then, the biofilms were exposed to CAP for 1, 3, 5 and 7 min. The biofilms were mechanically dispersed, and

the number of viable cells was determined by plating method. The recovery of each species was carried out by plating the resuspended biofilm in selective culture media (mannitol salt agar, cetrimide agar and Enterococcosel agar, respectively). The experiments were performed in triplicate in three different experiments ($n = 9$). A negative control (exposed only to helium flow and without ignition) was included for comparative purposes.

2.5. Morphologic Analysis of the Biofilms by Scanning Electron Microscopy

Multispecies biofilms exposed to CAP for 1, 3, 5 and 7 min were analyzed morphologically by scanning electron microscopy. After exposure to CAP, the biofilms were prepared by standard fixation protocol 2.5% glutaraldehyde solution in 0.1 M phosphate buffer (pH 7.4) for 24 h and serial dehydration in ethanol (Synth, 99.8%), for 10 min in each concentration [51]. After 24 h under room temperature, the biofilms were metallized in gold. The specimens were analyzed by scanning electron microscopy with Field-emission gun (SEM-FEG). The analysis was performed using a Tescan Vega 3 microscope with accelerating voltage of 10 kV and magnification of 10 k \times . Two fields per membrane were analyzed. The first was located at the central region of the membrane and the other at the peripheral region (approximately 2.5 mm from the center).

2.6. Cytotoxicity Evaluation

For evaluating the cytotoxicity of CAP, mouse fibroblastic cell line (3T3) were grown in DMEM (Dulbecco's Modified Eagle's medium) supplemented with 10% of inactivated fetal bovine serum (FBS), 100 IU mL⁻¹ of penicillin, and 100 μ g mL⁻¹ of streptomycin. After, 200 μ L of a suspension containing 3.6×10^4 cells/mL were transferred to the wells of 96-well plates. Plates were incubated for 24 h at 37 °C and in an atmosphere of 95% air and 5% CO₂. After, the culture medium was removed and 30 μ L of Hanks' Balanced Salt Solution with 10 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid were added. The cells were exposed to CAP for 1, 3, 5 and 7 min using the same parameters adopted for microbiological evaluations. The cell viability was determined immediately, 24 and 48 h after plasma exposure, using the MTT colorimetric method at 570 nm. For the analyses of later effects (24 and 48 h), the culture medium was immediately added after plasma jet exposure. The experiments were performed in triplicate in two independent experiments. The results were expressed as a percentage of viable cells (%), using the number of cells grown in wells without CAP exposure as the negative control.

The morphological grading of cytotoxicity, according to [52], was: 0 (not cytotoxic)—no reduction of cell growth; 1 (slight)—only slight growth inhibition observable; 2 (mild)—not more than 50% growth inhibition; 3 (moderate)—more than 50% growth inhibition observable; 4 (severe)—nearly complete or complete destruction of the cell layers.

2.7. Genotoxicity Evaluation

Genotoxicity was evaluated by static cytometry method for cell DNA ploidy analysis. NIH-3T3 cells were cultivated according to Felisbino et al. [53], with modifications. Cells (5×10^4 cells/mL) were seeded in 96-well plates in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% inactivated bovine fetal serum and penicillin/streptomycin 1% and maintained at 37 °C and 5% CO₂ for 24 h. Then, cells were washed once with Hanks' Balanced Salt Solution (HBSS) and 50 μ L of HBSS were added per well for CAP treatment. Cells were exposed to CAP for 1, 3, 5, and 7 min. A non-treated group was included for comparative purposes. After treatment, residual HBSS was aspirated, culture medium was added, and plates were maintained at 37 °C and 5% CO₂ for 24 h. Cells were released from plate with trypsin, fixed in 4% formalin and transferred to glass slides. Next, slides were stained by Feulgen's method and covered with cover glass.

Nuclear IOD (integrated optical density) was performed through static cytometry. Images were digitalized with Olympus QColor3 camera in 400 \times magnification. Image analysis were performed in a Carl Zeiss image system and KS300 software (Carl Zeiss, Oberkochen, Germany) in black and white with a Feulgen filter for 570 nm wavelength.

DNA index (DI) was obtained dividing resulting IOD by the mean of IOD from 20 lymphocytes used as diploid control. DI was classified as diploid (DI = 0.9–1.1), slightly aneuploid (DI = 1.2–1.4), moderated aneuploid (DI = 1.5–1.7), and severely aneuploid (DI > 1.8), based on Lima et al. [54]. IOD is equivalent but not identical to DNA content. A total of 100 cells were analyzed.

2.8. Data Analysis

Data was analyzed for normality using Shapiro Wilk test. After, data of cell viability of biofilms (CFU/mL) were compared by Kruskal Wallis followed by Dunn's post hoc test. The values of cytotoxicity were obtained by percentage analysis. For the genotoxicity assays, from the DI values x the number of cells, histograms of the frequency of distribution of the DI values by group were obtained. The analyses were carried out using GraphPad Prism, version 7.0 (Graphpad Software Inc., La Jolla, CA, USA). The significance levels were set at 5%, 1% or 0.1% ($p < 0.05$, 0.01 and 0.001), according to the statistical analyses' outcomes.

3. Results

3.1. Effect of CAP on Wound-Related Monospecies Biofilms

Monospecies biofilms formed by MRSA, *P. aeruginosa* or *E. faecalis* were exposed to CAP. Figure 1 shows the results for antibiofilm effect. The median value (CFU/mL) of the non-treated control for *E. faecalis* was 1.0×10^9 ; for treatment groups the median values (CFU/mL) were 5.8×10^7 (reduction of 1.2 log, $p < 0.05$), 5.2×10^7 (reduction of 1.3 log, $p < 0.01$), 1.4×10^7 (reduction of 1.9 log, $p < 0.001$) and 3.3×10^7 (reduction of 1.5 log, $p < 0.01$) after 1, 3, 5 and 7 min of CAP exposure, respectively. The median value (CFU/mL) of the non-treated control for MRSA was 6.4×10^8 ; for treatment groups the median values (CFU/mL) were 8.0×10^6 (reduction of 1.9 log, $p > 0.05$), 3.0×10^6 (reduction of 2.3 log, $p < 0.05$), 8.0×10^5 (reduction of 2.9 log, $p < 0.001$) and 1.4×10^6 (reduction of 2.7 log, $p < 0.001$) after 1, 3, 5 and 7 min of CAP exposure, respectively. The median value (CFU/mL) of the non-treated control for *P. aeruginosa* was 5.0×10^7 ; for treatment group the median values (CFU/mL) were 4.0×10^6 (reduction of 1.1 log, $p > 0.05$), 4.0×10^6 (reduction of 1.1 log, $p > 0.05$) and 2.2×10^5 (reduction of 2.4 log, $p < 0.05$) after 1, 3, 5 and 7 min of CAP exposure, respectively.

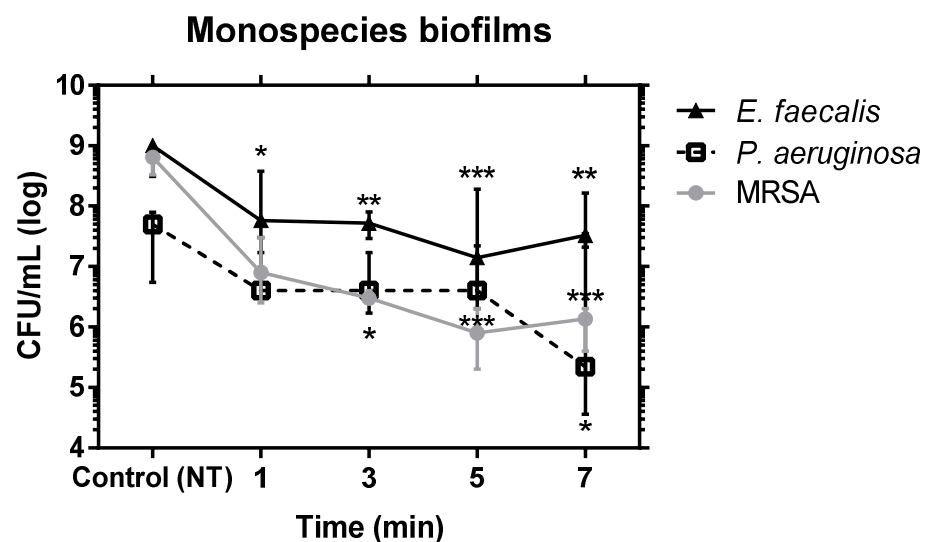


Figure 1. Median and interquartile range of MRSA, *P. aeruginosa* and *E. faecalis* viable cells (CFU/mL) recovered after exposure of monospecies biofilms to (CAP) for 1, 3, 5 and 7 min. Kruskal–Wallis test and Dunn's multiple comparison * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.2. Effect of CAP on Multispecies Biofilms

The effect of CAP on multispecies biofilms formed by MRSA, *P. aeruginosa* and *E. faecalis* can be observed in Figure 2. The median value (CFU/mL) of the non-treated control for *E. faecalis* was 2.3×10^9 ; for treatment groups the median values (CFU/mL) were 5.0×10^8 (reduction of 0.66 log, $p < 0.01$), 8.0×10^8 (reduction of 0.5 log, $p < 0.05$), 3.0×10^8 (reduction of 0.9 log, $p < 0.001$) and 7.0×10^8 (reduction of 0.5 log, $p < 0.05$) after 1, 3, 5 and 7 min of CAP exposure, respectively. The median value (CFU/mL) of the non-treated control for MRSA was 1.7×10^8 ; for treatment groups the median values (CFU/mL) were 1.1×10^8 (reduction of 0.2 log, $p > 0.05$), 3.7×10^7 (reduction of 0.66 log, $p > 0.05$), 2.0×10^7 (reduction of 0.9 log, $p < 0.05$) and 4.0×10^7 (reduction of 0.6 log, $p < 0.05$) after 1, 3, 5 and 7 min of CAP exposure, respectively. The median value (CFU/mL) of the non-treated control for *P. aeruginosa* was 3.0×10^7 ; for treatment group the median values (CFU/mL) were 6.0×10^6 (reduction of 0.7 log, $p > 0.05$), 5.0×10^6 (reduction of 0.8 log, $p > 0.05$), 1.0×10^6 (reduction of 1.5 log, $p < 0.001$) and 3.0×10^6 (reduction of 1.0 log, $p > 0.05$) after 1, 3, 5 and 7 min of CAP exposure, respectively.

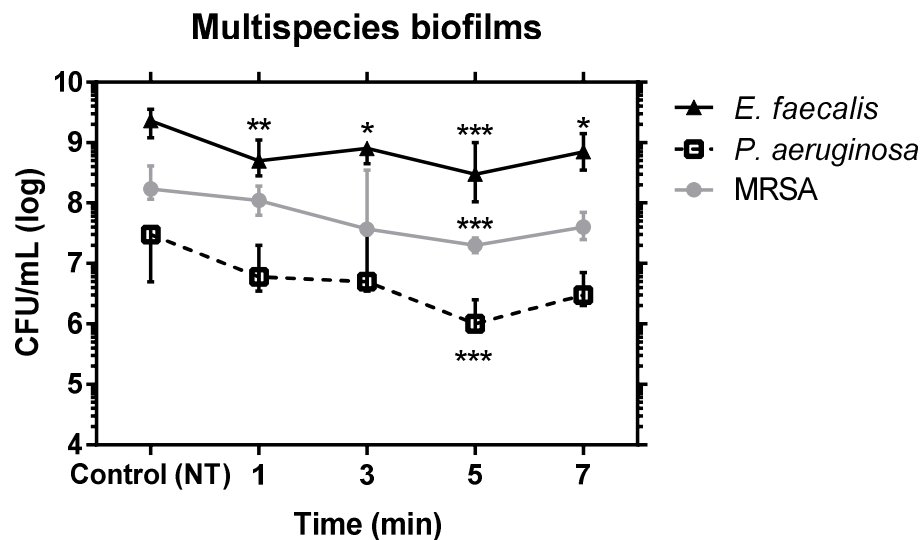


Figure 2. Median and interquartile range of MRSA, *P. aeruginosa* and *E. faecalis* viable cells (CFU/mL) recovered after exposure of multispecies biofilms to CAP for 1, 3, 5 and 7 min. Kruskal–Wallis test and Dunn’s multiple comparison post hoc test * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

3.3. Morphologic Analysis of the Multispecies Biofilms by Scanning Electron Microscopy

The biofilms morphology was analyzed by scanning electron microscopy (SEM) in order to confirm presence of biofilm and corroborate the reduction in cell viability observed in microbiological analyses. To assess the effects of CAP in biofilm, a polymicrobial culture comprised of a 1:1:1 ratio of MRSA, *E. faecalis* and *P. aeruginosa* was grown.

Figure 3 shows the micrographs obtained without treatment and after exposure to CAP. Untreated biofilm (control) showed the presence of high cell density of the cocci and bacilli. No differences were observed between the images from central and lateral fields.

In the groups exposed to CAP, SEM images showed dispersed biofilm clusters with moderate differences in the amount of microorganisms according to the analyzed field. After 1 and 3 min of exposure to CAP (Figure 3C1,D1), there was a greater presence of diplococci in the central field, the cell density in the biofilm was lower than in the non-treated control. In the lateral field, the cell density was higher and the presence of bacilli could be noticed (Figure 3C2,D2). After 5 min of exposure to CAP, the images showed a reduction in the cell density in the biofilm mostly in the center field. On the other hand, 7 min exposure to CAP leads to a visible biofilm reduction over the entire analyzed area.

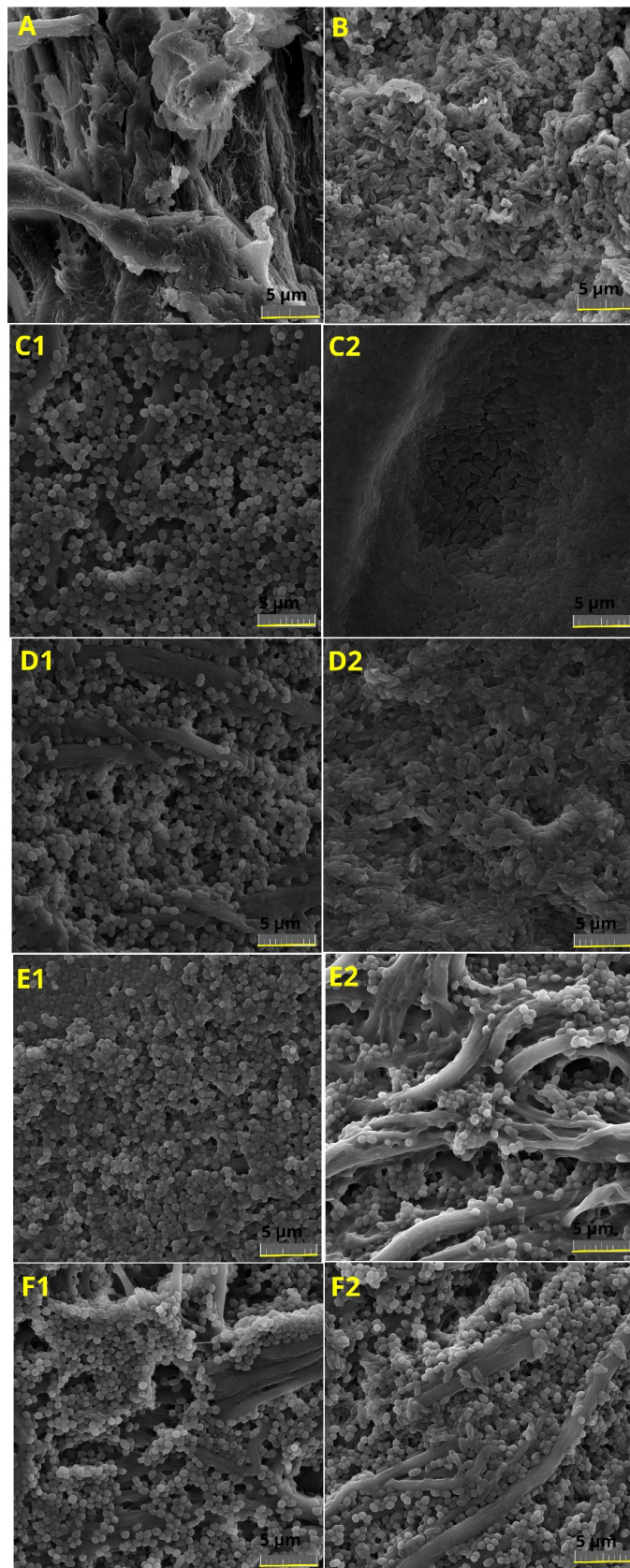


Figure 3. Scanning electron microscopy (SEM) of multispecies biofilms formed by MRSA, *P. aeruginosa* and *E. faecalis* non-treated or treated for 1–7 min: (A) Collagen membrane without biofilm, (B) negative

control (C1), biofilm after exposure to CAP for 1 min (central region), (C2) biofilm after exposure CAP for 1 min (lateral region), (D1) biofilm after exposure to CAP for 3 min (central region), (D2) biofilm after exposure to CAP for 3 min (lateral region), (E1) biofilm after exposure to CAP for 5 min (central region), (E2) biofilm after exposure to CAP for 5 min (lateral region), (F1) biofilm after exposure to CAP for 7 min (central region) and (F2) biofilm after treatment by CAP for 7 min (lateral region). Scale bare represents 5 μm .

3.4. Cytotoxicity

The 3T3 cells were exposed to the plasma jet for 1, 3, 5 and 7 min (Figure 4). The cytotoxicity immediately after exposure to plasma for all times was classified as Grade 0 (not cytotoxic), as the cell viability was 100% when compared to the unexposed control, except for 3 min, which was Grade 1 (slight), since the cell viability was 93.1% [52].

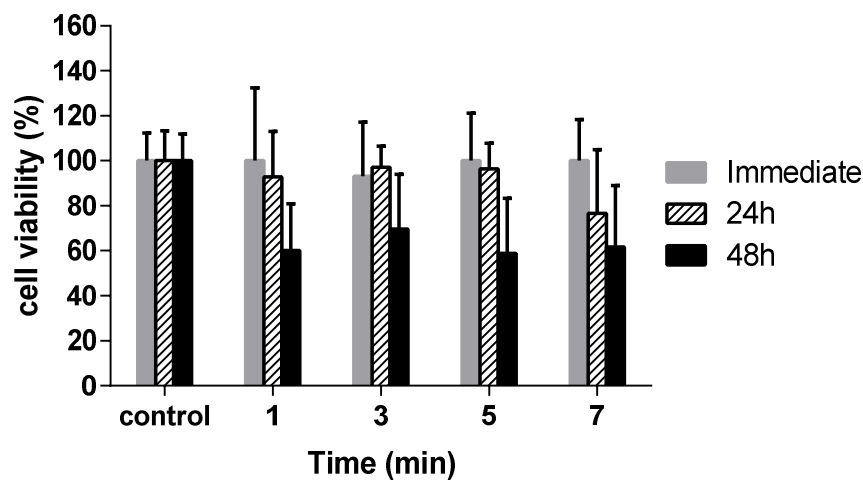


Figure 4. Cytotoxicity analysis of non-thermal plasma at atmospheric pressure expressed in cell viability. The 3T3 cells were exposed to CAP for 1, 3, 5 and 7 min. The cell viability was determined immediately (gray bar), 24 h (striped bar) and 48 h (black bar) after plasma exposure.

After 24 h of exposure to plasma, cytotoxicity was classified as Grade 1 (slight) for the exposure time for 1 to 5 min [52]. The exposure for 7 min was classified as Grade 2 (mild), since the cell viability was 76.6%. After 48 h of exposure to plasma, cytotoxicity was classified as Grade 2 (mild) for all times [52].

3.5. Genotoxicity

According to obtained results, all groups of 3T3 cells can be classified as slightly aneuploid ($DI \geq 1.2$). Non-treated group (NT) presented DNA index (DI) mean of 1.27. These results suggest that CAP was not genotoxic. Table 1 shows DI median, mean, and standard deviation (SD) of studied groups. Figure 5 represents the distribution histogram of obtained $DI \times$ number of cells for all analyzed periods of time.

Table 1. Median, mean and standard deviation (SD) of DNA index (DI) obtained by static cytometry of 3T3 cells' nucleus when treated with cold atmospheric plasma for 1 (P1), 3 (P3), 5 (P5), and 7 (P7) minutes. NT = non-treated group.

	P1	P3	P5	P7	NT
Median	1.24	1.33	1.30	1.27	1.24
Mean	1.25	1.32	1.31	1.29	1.27
Standard deviation (SD)	0.12	0.14	0.11	0.11	0.09

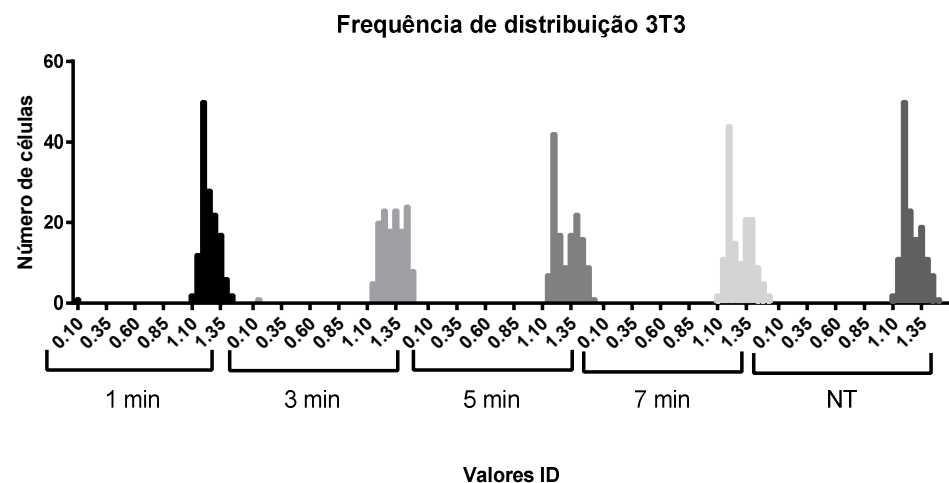


Figure 5. Histogram of distribution of DNA indices (ID) obtained \times number of corresponding cells 4.

4. Discussion

The outcomes of this study showed that He-cold atmospheric plasma significantly reduced the viability of wound-related mono and multispecies biofilms. *S. aureus*, *P. aeruginosa* and *E. faecalis* are opportunistic pathogenic bacteria which cause chronic infections due a number of virulence factors and can interfere with wound healing process, in particular when strains resistant to antimicrobials are involved [55]. To the best of our knowledge, this is the first report on the activity of CAP on wound related multispecies biofilms, formed by MRSA, *P. aeruginosa* and *E. faecalis*, by using a model of wound-related biofilm.

Although the main goal of this study was to evaluate the effect of cold atmospheric plasma in multispecies biofilms, experiments with monospecies biofilms were carried out in order to establish the operation parameters of the plasma device that generated antimicrobial activity against the tested microbial species. For this purpose and taking in account the cost of membrane used in the multispecies experiments, monospecies biofilms were grown in 96-well plates. We observed that the exposure to CAP for 5 min reduced in 3 log the viable cells of MRSA monospecies biofilms. This result corroborates the findings reported by Xu et al. [56], in which a plasma jet operating with 20 kV of voltage, frequency of 38 kHz, and He flow rate of 6.7 slm reduced in 3.6 log *S. aureus* biofilms after 10 min exposure. CAPs generated using other working gases also showed similar inhibitory effects. Matthes et al. [41] observed a reduction of 1.7 log in MRSA biofilms after repeated 20 s exposure to Ar plasma during 6 h. Besides, Wang et al. [57] observed a decrease of 4.67 ± 0.29 log in the viability of *S. aureus* biofilms after 10 min exposure to N₂ CAP.

We also detected a significant reduction in the viability of *E. faecalis* monospecies biofilm after 1 min exposure in the present study. The reduction varied from 1.2 to 1.9 log when compared to the untreated control group. This result is interesting, since *E. faecalis* monospecies biofilms appear to be resistant to other therapies. In the case of *E. faecalis* monospecies biofilm, it seems that other CAP generation setups lead to higher inhibitory effects when compared to He-CAP used in this study. Theinkon et al. [37] observed 3 log reduction in *E. faecalis* (ATCC 29212) biofilm viability after 5 min exposure to CAP generated by a surface micro-discharge reactor in open air operating with voltage of 3.5 kVp-p and a frequency of 4.0 kHz. It is important to highlight that in this study, differently than in ours, the treatment was applied indirectly (without direct contact between target and plasma) and the distribution of reactive species occurred by means of thermal convection and diffusion. Another study using CAP of Ar (98%) and O₂ (2%) observed 90% reduction in the viability of the 7-day biofilm of *E. faecalis* formed within the dental root canals of human teeth after 8 min of exposure [58].

For *P. aeruginosa* monospecies biofilms, we detected that He-CAP reduced in 2.4 log/CFU after 7 min of exposure. Alkawareed et al. [59] used a plasma jet with gas mixture (He/O₂) with voltage of 6 kV, frequency of 40 kHz and gas flow rate of 2 slm for treatment of *P. aerug-*

inosa and obtained a reduction of 4 log after treatment for 4 min. Besides, Gabriel et al. [36] reported a reduction of 5 log in *P. aeruginosa* counts after exposition for 90 s to microwave plasma jet (2.45 GHz) in air with flow rate of 5 slm, while we observed a reduction of 2.4 log after 7 min of exposure. However, it is important to mention that in both aforementioned studies the temperatures of CAP were above 40 °C, which could explain the higher reduction of the viable cells.

After determining the optimal parameters for monospecies biofilms, the effect of CAP on multispecies biofilms was studied. The study of mixed-species biofilms is relevant as, clinically, they can cause persistent infections and usually lead to worse infections [60–62]. The model of polymicrobial biofilm obtained on a porcine collagen membrane gave strong evidences that CAP can reduce the bacterial biofilm viability in complex models. The model described by Brackman e Coenye [8] for wound-related biofilms was selected because it mimics the conditions observed in wounds, by using a culture medium with a constitution close to the exudate and a nutrient flow diffusion from the bottom up. Besides, the model also mimics the air-wound-liquid interface that occurs in vivo [8].

The selection of the microbial species used to form the multispecies biofilms were based on their relevance in the context of chronic wounds infection. Previous studies reported that *S. aureus* and *P. aeruginosa* were the most prevalent species in wounds [4,11]. Besides, the presence of certain species, such as *E. faecalis*, seems to be related to worse clinical course of the wound [4,10,63].

In this study, *P. aeruginosa* was more susceptible to CAP in a multispecies biofilm, with reduction of 1.48 log. It is an unexpected result since previous report suggested that *P. aeruginosa* is one of the most benefited species in a mixed species culture, probably because it is present in a more protected niche of the biofilm [64].

It is known that CAP generates large variety of reactive species that can cause decontamination. Among them, ROS (e.g., O₃, O₂⁻, OH, O) are indicated as the major agent responsible for bactericidal effects of plasmas [65]. In plasma jets, the generated plasma plume excites and ionizes air molecules present in the surrounding ambient leading to formation of RONS [66]. The concentration of generated reactive species can be enhanced by adding molecular gases (e.g., air, O₂ or N₂) to the main carrier gas or applying a shielding gas curtain around the plasma plume [67]. In plasma jets, the produced reactive species are driven by the gas flow to the sample allowing short and long-living species to act on the treated surface, which can increase the treatment efficiency when compared to indirect methods. The plasma jet device used in the present study was operated with commercial low-grade purity helium that contains few impurities (mostly air) and ROS formation in the plasma plume was detected in previous studies [47,48]. Thus, the MRSA biofilm was significantly more susceptible than *P. aeruginosa* and *E. faecalis* under ROS-dominated conditions. Interestingly, the increased susceptibility of MRSA in a polymicrobial biofilm with *P. aeruginosa*, *E. faecalis* and *Klebsiella pneumoniae*, to RNS-dominated conditions were previously reported Modic et al. [31].

Our results showed that exposure to CAP for 1, 3, 5 and 7 min was not cytotoxic for fibroblasts 3T3 cells analyzed immediately after the exposure. Similarly, Borges et al. [45] found the cell viability to be higher than 80% immediately and 24 h after amplitude modulated cold atmospheric pressure plasma jet (AM-CAPPJ) exposure of Vero cells for 3 and 5 min. On the other hand, Borges et al. [45] showed that cell viability was maintained at around 80%, 48 h after AM-CAPPJ exposure, which was different from the present work where the cell viability has decreased to around 60% after 48 h. This could be explained by the internal cell signaling cascade that some reactive species may trigger as previously described [30,68,69]. Therefore, a potential cytotoxicity may be related to this lower cell viability after 48 h (late effects) in relation to the immediate time or 24 h, which could have side effects on cells. However, further chronic toxicity studies of CAP should be carried out. These late effects on fibroblasts could also exist for microorganisms and should be investigated in future studies.

Aiming the clinical application of CAP for wound care, it was extremely important to investigate whether plasma conditions would lead to DNA damage or not. In this study, all tested periods of treatments presented a DNA index between 1.2 and 1.33 (mean and median) which, according to the score, represents a slight ploidy, but taking into account that the CAP group was into the same score; median 1.24 and mean 1.27, we believe treatment was not genotoxic. Our results are coincident with others in the literature. Boxhammer et al. [70] observed no mutations, through EPRT assay, on V79 cells after 30 to 240 s of CAP treatment using a MiniFlatPlaSter device. Only a decrease in the proliferation rate was observed. Wende et al. [71] and Bekeschus et al. [72] tested the KINPen[®] through different methods of evaluation, and found no signals of genotoxicity due to plasma treatment. Wende et al. [71] assays used up to 180 s of CAP in non-cancer cells (HaCat and MRC5 cells) and melanoma cells (SK-Mel-147 cells) and did not find mutagenic changes with CAP treatment as well. They used HPRT1 mutation assay, micronucleus formation assay, and clonogenic assay, to confirm their findings. Kludge et al. [73] used the KINPen MED[™] to evaluate CAP genotoxicity using HET-MN model and also observed no genotoxic alterations on CAP treatment. In our study, static cytometry was chosen to calculate DNA index and, in that way, evaluate possible mutations caused by cold plasma treatment and found no genotoxic signals in cells exposed up to 7 min (420 s). Different CAP sources, cell types, CAP treatment conditions, and assay models reported make it difficult to compare results, but it seems that CAP safety has been proven by literature.

The inhibitory effect of CAP on wound-related multispecies biofilm with the low cytotoxicity and no genotoxicity observed in this wound-related multispecies biofilm study encourage *in vivo* studies to evaluate the effects of CAP on infected wounds that can also investigate additional anti-inflammatory and tissue repair inducing effects.

5. Conclusions

In conclusion, exposure to He-CAP for 5 min showed inhibitory effect on wound-related multispecies biofilms formed by methicillin-resistant *S. aureus* (MRSA), *P. aeruginosa* and *E. faecalis*, with low cytotoxicity and genotoxicity to mammalian cells mainly immediately after exposure. These findings point out the application of this innovative technology in the control of infected chronic wounds.

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