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Research Article

Repeated applications of cold atmospheric pressure plasma does not induce resistance in Staphylococcus aureus embedded in biofilms

Wiederholte Applikation von kaltem Atmosphärendruckplasma induziert keine Resistenzentwicklung bei Staphylococcus aureus in Biofilmen

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Abstract

Introduction: The increasing microbial resistance against antibiotics complicates the therapy of bacterial infections. Therefore new therapeutic options, particularly those causing no resistance, are of high interest. Cold atmospheric plasma is one possible option to eradicate multidrug resistant microorganisms, and so far no resistance development against physical plasma is known.
Method: We tested 6-fold repeated plasma applications on a *Staphylococcus aureus* strain embedded in biofilm and compared the reduction of the colony forming units between the different treatment periods to asses a possible development of resistance.
Result: For all treatment periods, the control biofilms were reduced by plasma in average by 1.7 $\log_{10}$ CFU, and decreased from 7.6 to 5.8 $\log_{10}$ (CFU/cm^2) within 5 hours. The results demonstrated that repeated plasma doses not induce resistance or habituation against plasma applied within short time periods.
Conclusion: The repeated application of cold plasma is a promising option for the treatment of infected wounds without the risk of development of resistance against plasma.

Keywords: cold atmospheric pressure plasma, resistance development

Zusammenfassung

Einleitung: Die ansteigende Antibiotikaresistenz von Bakterien erschwert die Therapie von durch sie verursachten Infektionen. Deshalb sind neue therapeutische Optionen, die keine bakterielle Resistenz induzieren, von größtem Interesse. Kaltes Atmosphärendruckplasma ist eine mögliche Option zur Eradikation multiresistenter Mikroorganismen, denn bis heute ist keine Resistenzentwicklung gegen kaltes Plasma nachgewiesen worden.
Methode: Wir untersuchten den Einfluss einer sechsfach wiederholten Plasmaapplikation auf einen in einen Biofilm eingebetteten *Staphylococcus aureus* Stamm und verglichen die Reduktion der Koloniebildenden Einheiten (KbE) zwischen den verschiedenen Applikationen, um eine mögliche Resistenzentwicklung festzustellen zu können.
Ergebnis: Bei allen Plasmabehandlungen wurde der Biofilm im Durchschnitt um 1,7 $\log_{10}$ KbE, d.h. von 7,6 auf 5,8 $\log_{10}$ (CFU/cm^2), innerhalb von 5 h reduziert. Damit konnte durch die wiederholte Plasmaapplikation innerhalb der Kurzzeitexposition keine Gewöhnung induziert werden.
Schlussfolgerung: Die wiederholte Applikation von kaltem Plasma ist eine aussichtsreiche Option zur Behandlung infizierter Wunden ohne das Risiko einer Resistenzentwicklung bei kurzzeitiger Anwendung.

Schlüsselwörter: kaltes Atmosphärendruckplasma, Resistenzentwicklung

Introduction

The development of bacterial resistance against antibiotics is an increasing challenge especially in health care [1], [2], [3]. In most situations, the microbial colonization of abiotic and biotic surfaces is accompanied with biofilm formation, which is an important pathogenic factor and one reason for direct or indirect support of the development of bacterial resistance [4]. Therefore, new therapeutic options to inactivate or to remove biofilms are of high interest.

Biofilms inhibit or block wound healing [5], [6]. In this context it is promising that the application of cold atmospheric argon plasma by the plasma source kinpen09 [7] is effective not only against biofilms [8], but induced complete healing of chronic wounds which did not respond to conventional and surgical treatment measures [9], [10].

The antimicrobial effect of plasma against a wide spectrum of bacteria including antibiotic resistant strains have been studied and reported in a number of experiments on solid agar plates [11], [12] and biofilms [13], [14], [15]. Currently, development of bacterial resistance against plasma is unknown and not expected, as its antimicrobial mode of action is physical and unspecific [16], [17]. The main target is the bacterial cell wall or membrane, which reacts with oxygen and nitrogen species in the plasma flow or in ambient liquid, resulting in lipid and protein oxidation or metabolic disruption [18]. However, plasma can modulate stress responses of microorganisms, which was demonstrated for *Bacillus subtilis* [19]. This may indicate the possibility for a potential bacterial habituation against physical plasma. Therefore, we investigated the influence of 6 repeated application steps of argon plasma on *Staphylococcus aureus* embedded in biofilms.

Methods

Microbial cultivation

The test organism *Staphylococcus aureus* ATCC 6538 was incubated for 48 h at 37 °C on polystyrene in a 96-well-microplate (Techno Plastic Products AG, Trasadingen, Switzerland). The growth medium was similar to an artificial wound medium (minimal essential medium with 10% fetal bovine serum; GIBCO-Invitrogen, Darmstadt, Germany) [20], [21]. 80 µl of the inoculum at a concentration of 10^8 CFU/mL was used for four resp. five wells in 6 separate microplates for plasma treatment and control specimen. Additionally, a negative control was carried along, which was not treated with plasma at any time period. Before plasma application, the biofilms were washed with 90 µL Dulbecco's buffered saline solution (PAA Laboratories/GE Healthcare Europe GmbH, Munich, Germany) one time.

Plasma treatment

A spatial afterglow cold plasma was generated by a radio frequency plasma pen (kinpen09®, neoplas GmbH, Greifswald, Germany) [7], using argon (99.995% pure) as carrier gas with a controlled gas flow rate at 5 sLm (standard litre/min) (MKS Instruments, Munich, Germany). The input power was set at 1.1 MHz at 2–6 kVpp with a maximal input DC power of 3.5 W to the hand-held unit, resulting in a mean heat output of approximately 300 mW on the target surface [7].

During the treatment the generated plasma jet was directed at the treated surface open to the indoor air. For all experiments, the plasma pen was fixed in a computer-controlled x/y/z table (modified EDX-20, Roland DG, Westerlo, Belgium) above the biofilm containing microplate [9]. The distance between the nozzle of the plasma pen and the biofilm was 10 mm. After 20 sec plasma treatment of each biofilm and each microplate, 80 µl medium was transferred in all biofilm-containing wells and incubated again for 1 h at 37 °C.

All 6 microplates prepared with biofilm were plasma-treated. A separate plate with 5 biofilm wells without treatment served as negative control and further 5 biofilm wells served as control to check the stability of the plasma efficacy between the first and the last plasma exposure. A final separate plate served as plasma control to control for possible changes in plasma efficacy, and control biofilms for the first CFU assay of the biofilms in microplate #1 at beginning of the experiments. After 1 hour of incubation of the remaining 5 biofilm prepared microplates, all biofilms were plasma-treated, with exception of the control biofilms on the microplate #2 at time 1 hour. The same procedure was performed for the positive plasma controls. This procedure was repeatedly performed 6 times in sequential duplicates (n=9). At time 6 hour, all biofilm-covered wells were treated by plasma, again, with exception of the negative control (Figure 1).

Analysis

After exposure, biofilms were dispersed in an ultrasonic bath (130 W, Branson 2510, Emerson Technologies GmbH & Co. OHG, Dietzenbach, Germany) for 20 min. The antimicrobial effect was determined as the difference in the number of CFU in the suspension as described before [14]. The CFU of the treated sample (vs) were compared with the mean of the non-treated control sample (mc) of each test run. The reduction factor (RF) was defined by the formula:

$$RF=\log_{10}(mc)-\log_{10}(vs)$$

The standard deviations (±) and p values (α=0.05) were calculated based on the RFs in $\log_{10}$ (CFU). Statistical differences were analyzed using the Kruskal-Wallis test, followed by the Dunn's Multiple Comparison Test (Prism, GraphPad, USA).

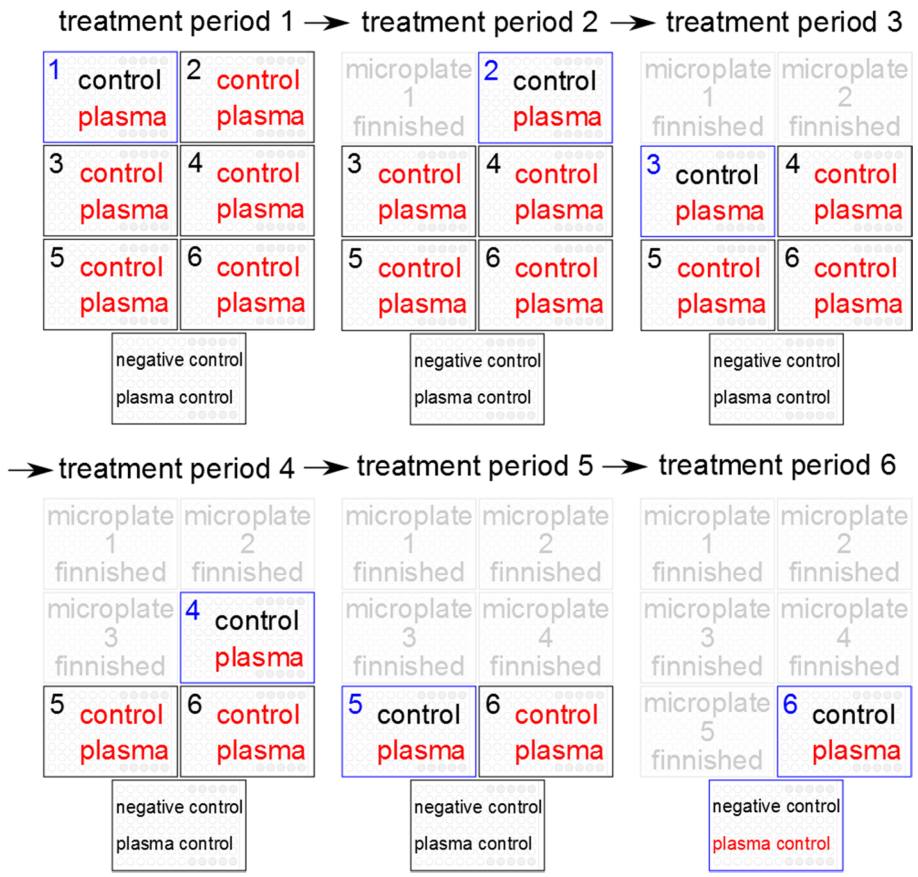

Figure 1: Treatment regimen of the 6 biofilm prepared 96-well-microplates for each treatment period. Note plasma treated control biofilms in black and plasma treated biofilms in red letters, blue microplates were used to determine the colony forming units (CFU).

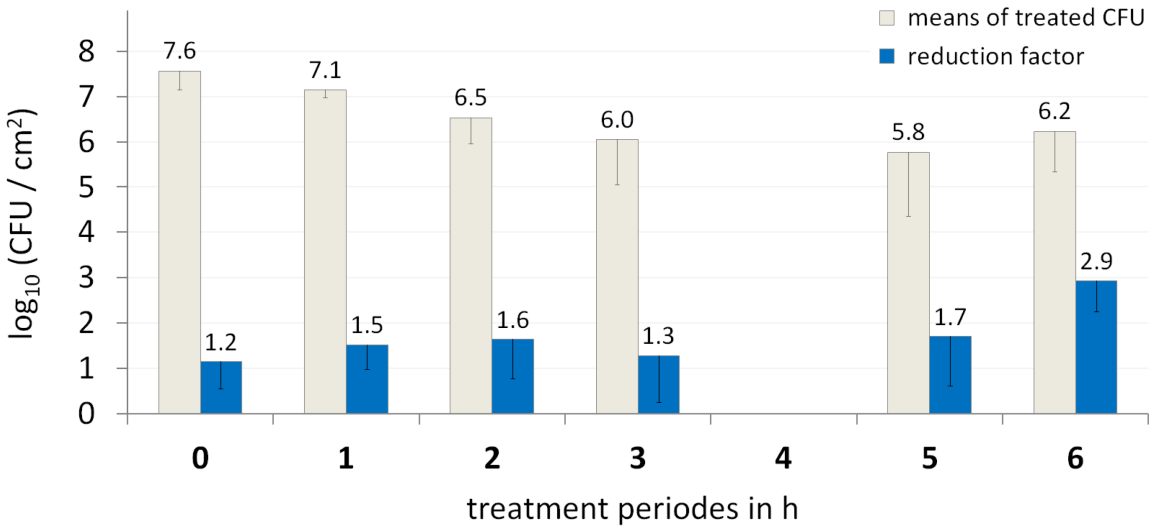

Figure 2: Mean values of the CFU of *S. aureus* ATCC 6538 of the control biofilms (grey bars) and of the reduction factors after argon plasma treatment (dark blue bars) of each treatment period after 0, 1, 2, 3, 5 and 6 h; error bars show the standard deviation (each n=9).

Results and discussion

This is the first study investigating a potential resistant development in Gram psositive bacteria embedded in biofilm. The analysis was performed after 0, 1, 2, 3, 5 and 6 hour. For all treatment periods, the control biofilms were reduced by plasma in average by 1.7 $\log_{10}$ CFU, and decreased from 7.6 to 5.8 $\log_{10}$ (CFU/cm^2) within 5 hours (Figure 2). The comparison of the RFs at times 0, 1 and 3 h showed statistically significant differences compared to the RF after the 6th hour ($p<0.05$). The negative control (mean 7.7 ±0.4 $\log_{10}$) and the positive plasma control

(RF 0.8±0.4) significant differences to their initial inoculums at start ($p<0.05$). Conclusively, the plasma efficiency was constant for the 6 treatment periods.

At every hour, the bacteria cells could replicate themselves and had the theoretical possibility to adapt to the plasma-induced stress. By comparing the RF of each treatment period, a difference of the susceptibility against plasma should be observable. After 4 times of plasma treatment (3rd hour) the replication-rate and the reduction-rate due to plasma were kept a level of approximately 6 $\log_{10}$ (CFU/cm^2). Within the 6 times of hourly plasma treatment no decreased susceptibility of *S. aureus* against the antimicrobial components and the stress caused by 60 sec of argon plasma treatment was observable. After the 6th treatment the resistance against plasma decreased slightly, but not significantly. However, the reduction factor increased trendwise, while not significantly. Thus, a "gradual habituation" to the plasma reactive compound was not detectable in this experiment for the investigated *S. aureus* strain.

Conclusively, a repeated plasma application with a stable reduction-rate over time is expected. These results are in concordance with another study where the study group tested the possible bacterial resistance against plasma treatment on *Escherichia coli* and *Enterococcus mundtii*. There, induction of resistance against plasma was also not detectable [22].

A further detail of interest was that it was demonstrated that plasma has no remanent antibacterial effect. To ensure an antimicrobial long term effect to avoid a microbial recovery, the plasma treatment could be combined with antiseptics with remanent efficacy, such as used for the treatment of chronic wounds with application of polihexanide or octenidine after plasma treatment [9], [10], [23].

Conclusion

The antimicrobial effect of plasma on *S. aureus* is stable for repeated application doses. Since no induction of bacterial resistance against plasma treatment was observed, this method may be an option for the treatment of infected wounds. Because the antimicrobial effect shows no remanent effect, a repeated application seems to be required or a combination with topical antiseptics. Repeated plasma applications for a higher number and longer time period should be investigated in further studies.

Notes

Competing interests

The authors declare that they have no competing interests.

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