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Surface Microdischarge Plasma for Disinfection

T. Shimizu,[a,b,*] V. Lachner,[b,c] & J.L. Zimmermann[a,b]

[a]terraplasma GmbH, Garching, Germany; [b]Max-Planck Institute for Extraterrestrial Physics, Garching, Germany; [c]Division of Neuropathology, Institute of Pathology, Technical University Munich, Germany

*Address all correspondence to: T. Shimizu, terraplasma GmbH, Lichtenbergstr. 8, Garching, Bavaria, Germany; Tel.: 49-89-5484-2270; Fax: 49-89-5484-2279, E-mail: shimizu@terraplasma.com

ABSTRACT: Cold atmospheric plasma (CAP), with its antibacterial, antiviral, and antifungal properties, may offer different applications for medicine and medical technology. Wound care, the treatment of chronic or acute wounds, reflects one of these possible applications, because CAPs can inactivate different kinds of microorganisms, including antibiotic-resistant strains. In this article, we describe the way in which surface micro-discharge (SMD) plasma was investigated to evaluate a safe therapeutic window for wound treatment. We found that SMD plasma provided a significant bactericidal effect without harming skin cells. For testing the bactericidal efficacy of the SMD electrode, we used *Escherichia coli* as the test strain. To evaluate the response of plasma-treated eukaryotic cells, we used primary human dermal fibroblasts. As reported earlier, SMD plasmas have a unique characteristic in that the plasma chemistry produced in a confined volume can be varied by changing the input power into the plasma: low-input power produces a more reactive oxygen species–based chemistry and high-input power a more reactive nitrogen species. Using this characteristic, bactericidal effect and cell viability were investigated for a wide range of chemical plasma cocktails with different compositions and concentrations of mainly reactive oxygen and nitrogen species. Our results clearly reveal therapeutic plasma conditions wherein a high bactericidal effect and significant cell viability can be achieved simultaneously.

KEY WORDS: cold atmospheric plasmas, disinfection, reactive species, wound treatment

I. INTRODUCTION

It is well known that wound healing processes are affected by bacteria and its products.[1] Tissue responds with an infection if the number of bacteria present is higher than a certain threshold.[2] In addition, higher levels of bacteria not only result in infection but also inhibit wound-healing processes.[3] Therefore, it is believed that healing can be improved by reducing the bacterial load in wounds.[4]

Earlier studies demonstrated that cold atmospheric plasmas (CAPs) can inactivate microbes very efficiently.[5–8] With this characteristic, a clinical study was carried out to reduce the bacterial load of chronic wounds with the use of a microwave plasma torch.[9] This study showed that the bacterial load in wounds decreased significantly after CAP treatment, and the treatment did not induce side effects or allergic reactions.[10,11] Similar results with another CAP device—a dielectric barrier discharge plasma generator—were obtained in a small clinical study of chronic venous leg ulcers.[12]

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CAPs are partly ionized gases that produce a reactive plasma cocktail composed of electrons, ions, excited atoms and molecules, reactive species (such as O_3, NO, OH, and NO_2), and ultraviolet (UV) light. Among these, reactive species are considered to be the main players involved in the observed bactericidal effects in wounds.[13] Nevertheless, it is still under intensive investigation how different CAPs react with different tissue and eukaryotic cells.

We would like to point out that different plasma systems generate different plasmas, that is, different plasma cocktails. Depending on the plasma device, gas used, and applied plasma production parameters such as voltage or frequency, plasma treatment time, and nature of the application, the reactive plasma cocktail varies in composition and concentration of the respective species. Consequently, different plasma devices produce different plasmas and in general cannot be compared. It was also found that by changing only the plasma production parameters (voltage and frequency, i.e., the power consumption), the plasma cocktail produced by a so-called surface micro-discharge (SMD) electrode in a confined volume could be changed drastically.[14,15] For a low-power consumption, the plasma cocktail contains mainly reactive oxygen species, and by increasing power consumption reactive nitrogen species dominate the cocktail.[16] This shows that even one plasma device can produce different plasma cocktails and that plasma production parameters must be controlled carefully.

Due to its high inactivation properties, the SMD electrode is the appropriate technology for the treatment of acute and chronic wounds.[7,17–20] No resistance against SMD plasma treatment was observed.[21] Furthermore, SMD plasma treatment did not induce mutagenicity in V79 Chinese hamster cells under the investigated conditions.[22] In our study, we prolonged the investigations carried out with SMD technology and investigated the effect of different operating conditions (different plasmas) on eukaryotic and prokaryotic cells. We aimed to investigate and evaluate a safe therapeutic window where a high bactericidal effect is present without harming primary human dermal fibroblasts.

II. EXPERIMENTAL SETUP

As the CAP source, we used an SMD plasma electrode. The electrode consisted of a 0.5-mm-thick Al_2O_3 plate sandwiched by a copper planar electrode of 25 mm in diameter and a structured electrode (a wire mesh with a separation of 5 mm), as shown in Fig. 1. The structured electrode was grounded through a 0.1-μF capacitance for the power measurement.[23] A high voltage of 9 kV_{pp} with a sinusoidal waveform was applied using a function generator and a high-voltage amplifier (Trek Inc., model 10/10B) for plasma production on the side of the structured electrode. To change the plasma products (the chemistry), we changed the frequency of the applied voltage from 60 Hz to 2 kHz.[8] To measure O_3 in the plasma gas volume and inactivation tests using bacteria, plasma gas volume was confined by the SMD electrode in a quartz glass tube (30 mm in diameter and 20 mm in height) and a plate, as shown in Fig. 1a. For the treatment of human dermal fibroblasts, a confined plasma gas volume was created by the electrode and a well of a six-well plate as shown in Fig. 1b.

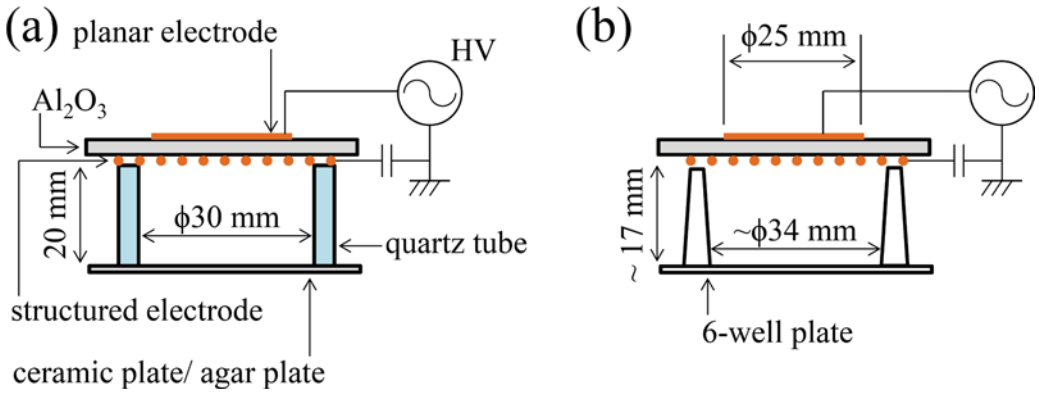

FIG. 1: Experimental setup using the SMD electrode. (a) Plasma gas volume is confined with an electrode, quartz glass tube, and plate. This configuration was used for O_3 measurement and bacteria treatment. (b) Plasma gas volume is confined within one well of six-well plate for the treatment of fibroblasts. HV, High voltage.

In general, UV power produced by SMD plasmas is negligibly small. Using the SMD electrode, the maximum UV power generated was 50–80 nW/cm^2, measured using a Hamamatsu C8026 UV power meter. We measured O_3 concentration using absorption spectroscopy and a UV light at 254 nm wavelength produced by an Hg/Ar lamp. As shown in Fig. 1a the configuration used for this measurement incorporated a ceramic plate. At a height of 10 mm from the ceramic plate, ozone concentration was measured using ambient air of 20°C–23°C with 40%–50% relative humidity. When cell cultures or agar plates are treated with SMD plasma, the humidity in the confined volume presumably increases. To simulate this condition, the O_3 evolution was measured for different input powers and humidity values. These measurements were repeated at least three times. The shown data is averaged data with the standard deviation.

To evaluate the bactericidal efficacy of the SMD plasma, a suspension of *Escherichia coli* (DSM 1116) in phosphate-buffered saline (PBS) with a density of ~1×10^8 cells/mL was prepared as a master bacterial suspension. A volume of 100 µL of this suspension and its dilution was distributed homogeneously on Mueller-Hinton agar plates and dried for 30 min in ambient air. The prepared bacteria samples were treated for 15, 30, 45, and 60 s with CAP, using the configuration shown in Fig. 1a. We counted colony forming units corresponding to survived bacteria 16 hr after incubation at standard conditions.

To investigate the effect of CAP on eukaryotic cells, we used a primary cell line of normal human dermal fibroblast cells from adult donors (PromoCell; Heidelberg, Germany). All cells were maintained according to standard protocols with McCoy's 5A Medium (Sigma-Aldrich), supplemented with 10% fetal calf serum (FCS) and 1% antibiotic/antimycotic solution until the 10th passage under standard cell culture conditions (5% CO_2 and 37°C). To investigate cell viability, 1×10^5 cells were seeded in six-well plates. Just before CAP treatment, the medium was removed until no visible liquid remained. Cells were CAP treated for 30, 60, 90, and 120 s using the configuration

shown in Fig. 1b. After treatment, we immediately added fresh medium containing 10% FCS to the plates. Controls were kept without medium in ambient air for 120 s. The cells were incubated for 48 h at standard cell culture conditions and then washed, trypsinized, and counted with a hemocytometer. All CAP treatments and controls were performed in triplicate. We evaluated the averaged values with standard deviation.

III. RESULTS AND DISCUSSION

Figure 2a shows the ozone concentrations of C_{O3} as a function of time t measured under "dry conditions," where the initial gas in the confined volume was composed of ambient air. When input power was low, C_{O3} increased monotonically. By applying an input power of > 430 mW/cm^2, C_{O3} peaked after 10–30 s upon SMD plasma ignition and then decayed to less than the detection limit. This transient behavior is quantitatively consistent with that described in our previous report.[8] The observed O_3 destruction was not due to thermal decomposition but rather to a transition from an O_3 to an NO_x (e.g., NO and NO_2) mode.

When 50 µL of distilled water was distributed on the ceramic plate in the confined volume, measurement of C_{O3} showed reduced C_{O3} and a longer lifetime of O_3 than those of the dry condition without water shown in Fig. 2b. By adding distilled water, the humidity in the confined volume increased. Lower C_{O3} can be explained by a quick reaction of the singlet O atom and a water molecule.[24,25] Reduced concentrations of O atoms lead to lower O_3 concentrations. We observed longer lifetime of O_3 especially when the applied power was 430 mW/cm^2. O_3 was not destructed within 120 s, although it was quenched within 90 s without adding water (dry condition). It seems that the reactive species generated from water vapor reacts with NO_x and prevents the destruction of O_3.

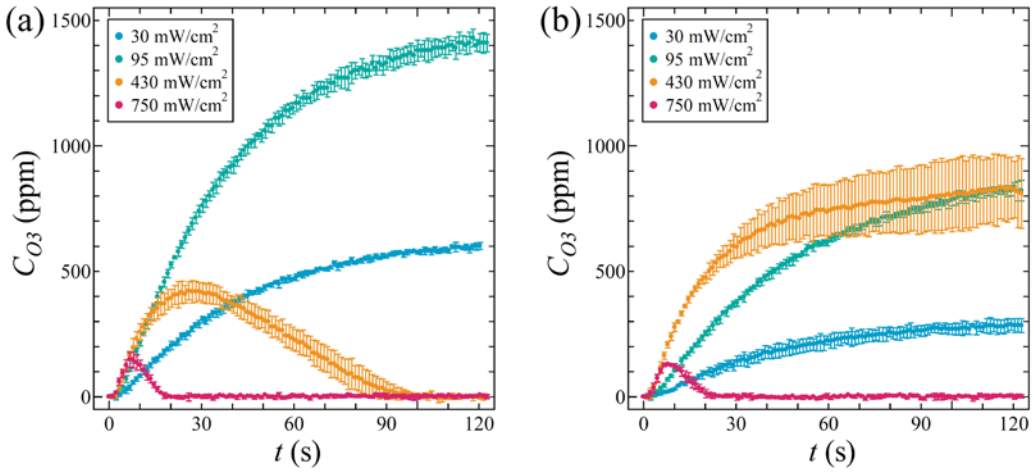

FIG. 2: O_3 concentrations of C_{O3} as a function of time t. (a) "Dry condition": C_{O3} was measured using ambient air in the confined volume; (b) "wet condition": C_{O3} was measured with 50 µL of distilled water on a plate.

For example, the reaction rate constant of OH with NO and NO_2 is 100 times higher than the reaction rate constant with O_3. It is important to note that almost the same C_{O3} evolutions were observed when PBS was applied in place of distilled water (data not shown).

Figure 3 shows the bactericidal efficacies of SMD plasma using *E. coli* on agar. When input power was 30 mW/cm^2, we observed almost no bactericidal effect even after long plasma treatment times of 60 s. Higher bactericidal effects were observed for higher input powers. After 60 s, we achieved almost 5 log reduction (detection limit) with all of the investigated input powers except for the lowest power of 30 mW/cm^2.

A simple indicator for cell viability is the number of surviving cells that are found 48 hr after CAP treatment. In this study, we examined this viability for treatment times of up to 120 s. Figure 4 depicts the numbers of cells treated with SMD plasma for different input powers. The results show no large differences in total cell amounts among all input power conditions. Nevertheless, data clearly show that longer treatment time *t* and higher input power result in lower survived cell numbers.

Because O_3 (produced in the plasma cocktail) is an important agent for inactivating bacteria, we plotted the observed bactericidal effect by CAP shown in Fig. 3 against applied O_3 doses; Fig. 5 shows the bactericidal effect as a function of O_3 dose. In general, higher O_3 dose yields higher bacterial log reductions. However, the bactericidal effect is strongly dependent on the input power at ~0.5×10^4 ppm·s in ozone dose. We observed a higher log reduction with higher power input, whereas almost no reduction was observed at a low power of 30 mW/cm^2. This implies that the other reactive species produced by the plasma play an important part in the inactivation of bacteria. For

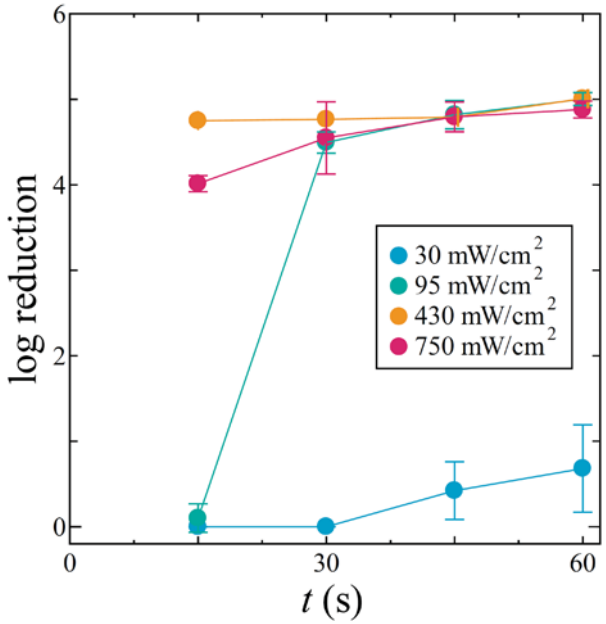

FIG. 3: Bactericidal effect of SMD plasma for different input powers

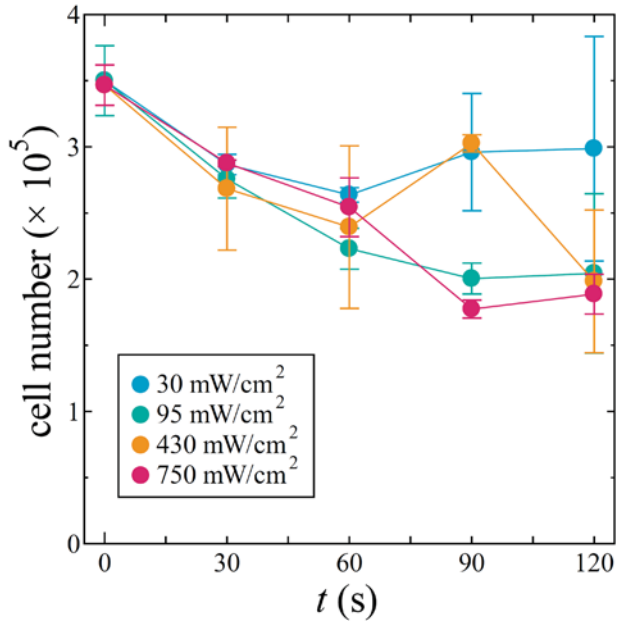

FIG. 4: Cell numbers 48 hr after CAP treatment. Cells were treated for 30, 60, 90, and 120 s. Controls at $t = 0$ s were untreated cells. The number of the cells were counted using a hemocytometer.

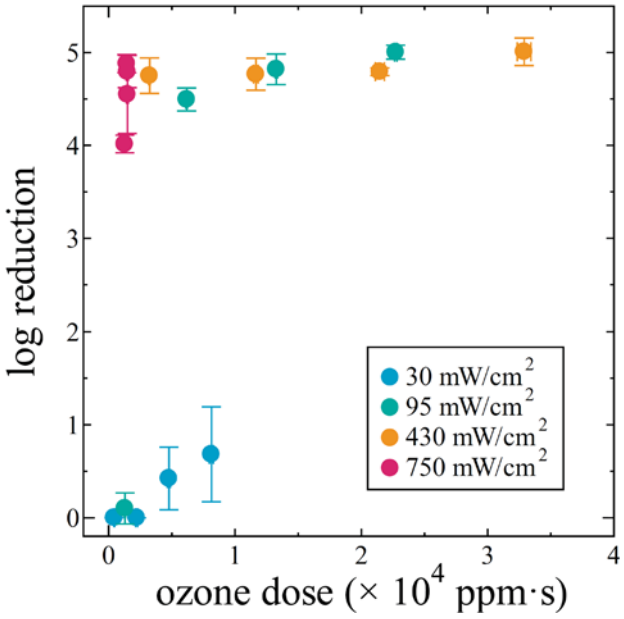

FIG. 5: Bactericidal effect as a function of O_3 dose

our experimental setup, the distance between electrode and agar surface was set to 20 mm. Consequently, only long-lifetime reactive species can reach the surface of the agar plates inoculated with bacteria. Other than O_3, produced long-lifetime species are N_2O, NO_2, HNO_3, N_2O_5, and H_2O_2.[26] Until now, the influence of the respective plasma species on bacterial efficacy is still under extensive investigation to determine which species have major roles in inactivating bacteria. At this stage, it should be noted that for SMD plasmas produced in a confined volume, one observes either O_3 or NO_2 but not both at the same time.[16]

To investigate the influence of O_3 dose on cell viability, both components were plotted against one another, as shown in Fig. 6. Cell viability was calculated using the number of cells in the control sample (t = 0 s). Except for the highest input power of 750 mW/cm^2, higher O_3 doses led to lower cell viability. As noted above, we removed the medium normally covering the cells in the cell culture shortly before plasma treatment until no visible liquid remained. However, a thin layer of liquid was of course still present during CAP treatment. The products produced by the reaction of O_3 and water, such as HO_2, could be involved in the reduction of cell viability.[27,28] When input power was 750 mW/cm^2, cell viability decreased regardless of O_3 dose. At this input power, ozone was present for only 20 s and reactive nitrogen species such as NO_2 were produced consecutively. The contribution of reactive nitrogen species must be investigated to understand the response of cells toward these species.

CAP treatment is a painless procedure to decrease the bacterial load in wounds, independent of the type of bacteria present.[10,11] Thus, CAP treatment reflects a suitable

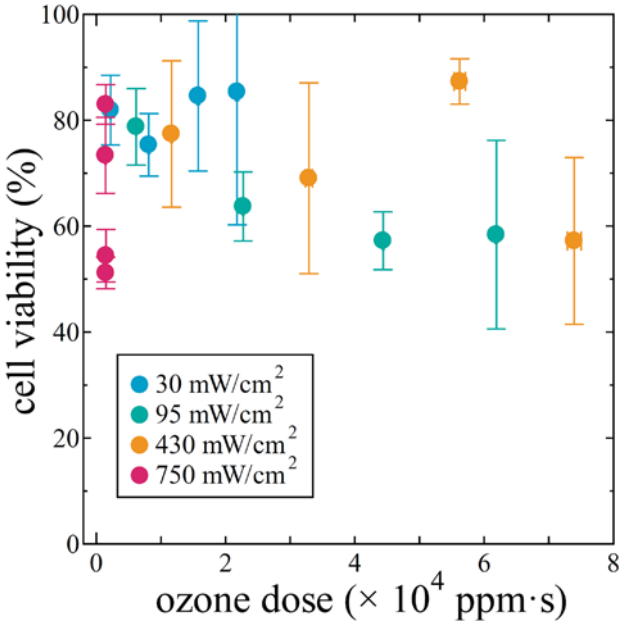

FIG. 6: Cell viability as a function of O_3 dose

therapy for the treatment and care of wounds. In this study, we discuss a safe therapeutic window for wound care using SMD plasma from the viewpoint of its bactericidal properties and its effect on cell viability of primary skin cells. In Fig. 7, bactericidal efficacy is plotted against obtained cell viability of plasma-treated skin cells. The bacterial log reduction for treatments of 90 and 120 s was extrapolated from the data shown in Fig. 3. In Fig. 7, three groups of data points can be observed. The first group reflects points of high cell viability of 80%–90 % and low bactericidal properties. These data were obtained for an input power of 30 mW/cm^2. In the second group, a high bactericidal effect of an ~ 5 log reduction was obtained, but cell viability was low (50%–70%). These data reflect the results of high input power or/and a long treatment times. The third group shows the data corresponding to a high bactericidal effect of ~ 5 log and high cell viability of ~80%. These data are associated with an input power of 430 mW/cm^2 and treatment times of 30–60 s. On the basis of these results, we can conclude that for certain operating conditions, a safe therapeutic window of high bactericidal effect and high cell viability can be simultaneously achieved. Moreover, it is evident that a small change in operating conditions can cause a huge change in biological reactions using CAP.

The tests carried out in this study are prerequisites for evaluating safe operating conditions of SMD plasmas. Further safety tests are necessary such as mutagenicity tests[22,29,30] using the therapeutic conditions mentioned above and investigations of the effect of CAP on deeper layers of the skin.[31] Nevertheless, the results obtained in this study provide a good basis for the evaluation of a safe therapeutic window using SMD

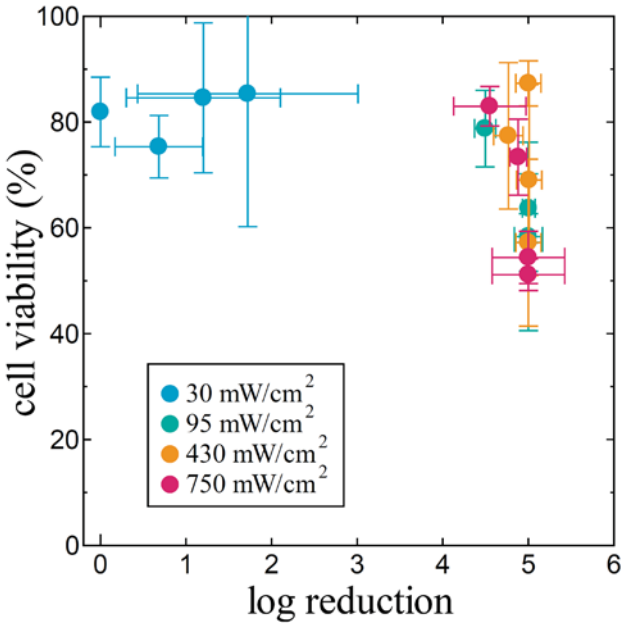

FIG. 7: Relationship between bactericidal effect and cell viability of SMD plasma. A therapeutic window exists with high bactericidal effects and high cell viability.

plasma and show how sensitive eukaryotic and prokaryotic cells react to changes in applied plasma chemistry.

IV. SUMMARY

As reported earlier, the results show that the plasma cocktail produced by SMD plasma can be changed drastically with variations in input power. We intensively studied the effects of different plasma cocktails on eukaryotic (primary dermal fibroblasts) and prokaryotic (*E. coli*) cells. We showed that the response of eukaryotic and prokaryotic cells to different plasma cocktails changes drastically. In addition, our results show a range of plasma conditions that yield high bactericidal effects and simultaneously high eukaryotic cell viability. The definition of this safe therapeutic window is a prerequisite for the application of SMD plasma *in vivo*. Nevertheless, further investigations using SMD plasma are still required and must be conducted before clinical studies on humans.

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