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In vitro killing of mycobacteria by low temperature atmospheric pressure plasma and dielectric barrier discharge plasma for treatment of tuberculosis

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ABSTRACT

Background: To prove the efficacy of cold atmospheric plasma (CAP) to inactivate mycobacteria in vitro, we tested the susceptibility of important mycobacterial species to evaluate potential alternative treatment options.
Methods: We tested 8 different mycobacterial species *M. tuberculosis*[2] (MTB*)* (resistant against isoniazid (INH)), *M. marinum*, *M. ulcerans*, *M. abscessus*, *M. chelonae*, *M. africanum*, *M. terrae* and *M. avium* against two typical CAP devices, a low temperature atmospheric pressure plasma jet (APPJ) and a dielectric barrier discharge plasma (DBD). The tests followed a modified standard procedure by comparing obtained inhibition zones after irradiation of 3, 15, 30 and 60 s on selective (Löwenstein-Jensen) medium.
Results: Irradiation by both sources proved high antimycobacterial killing effectiveness against all species including the INH-resistant strain of *M. tuberculosis* and a set of most important mycobacterial pathogens causing mycobacteriosis. Plasma irradiation $< t = 60$ s resulted in significant microbiocidal reduction of mycobacteria of the MTBC-complex and important nontuberculous mycobacteria (NTM) in vitro. DBD and APPJ plasma killed all isolates on Löwenstein-Jensen medium.
Conclusion: Plasma treatment (both sources) may serve as a future option for treatment of skin tuberculosis evolved from *M. tuberculosis* (lupus vulgaris), NTM and leprosy and also for surface decontamination after suspected contact with acid fast microbes.

1. Introduction

According to the US Centers for Disease Control (CDC), approximately one-third of the world's population is currently infected with tuberculosis (TB) and every year, there are almost nine million new cases of infection with approximately two million deaths per year due to TB infection. HIV and TB are specially related: They are syndemic, which is defined as the convergence of two or more diseases that act synergistically to magnify the burden of one or both diseases. Without adequate control of the TB-HIV syndemic, the long-term TB elimination target set for 2050 will not be reached. Thus novel approaches for diagnosis, treatment, and prevention of both HIV and TB [21] were urgently required.

Plasma medicine is an expanding focus and offers new aspects of therapy combining potent physically acting parts like UV, IR, reactive species and particles and nowadays many successful treatments of different illnesses have been described. Non-thermal atmospheric pressure plasma has been introduced in medical and biological applications since it demonstrates potent antimicrobial in vitro efficacy as well as medically important biochemical effects [1–3]. Within the last years, first results of clinical plasma applications were reported to treat diverse skin and soft tissue infections like bacterial dermatitis, chronic ulcer wounds and eye lid infections but also severe pulmonary tuberculosis [4,5]. Also in hospital hygiene, a cold plasma based decontamination device for skin disinfection was presented by Morfill and coworkers (HandPlaSter®) and showed high disinfection efficacy in

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[2] MTB: Mycobacterium tuberculosis.

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preliminary studies [6].

In our previous work we were able to show that the plasma sources-APPJ and DBD- were highly effective killing several bacterial and fungal species [7] on agar after 3 s up to 30 s treatment without exception. Together with similar published data by other groups and in combination with non- critical data from risk assessment it can be deduced that plasma could also be effective in preventive medicine to disinfect skin and other environmental surfaces known as risk factors for germ distribution. By this way plasma could play a relevant role as first "physical antisepsis". Meanwhile clinical applications confirm not only good CAP results in vitro but CAP also seems a useful support in the treatment of chronic wounds with involvement of nosocomial multidrug resistant pathogens like MRSA, ESBL, VRE and others [8–10].

Even with the help of highly effective antituberculous drugs cutaneous tuberculosis with about 1.5% of all extra pulmonary tuberculosis constitutes a significant medical problem. In the majority of cases it is caused by *M. tuberculosis*[3] (MTB) and, rarely, by *M. bovis*. It accounts for 0.1–0.9% of the total dermatology out-patients in India [11–13] and its incidence in children has varied in different studies depending on the geographical region. In older surveys from Hong Kong, up to 50% of all cases of cutaneous TB were found in children, although now the overall incidence of the disease is very low in that country [14,15]. In India, the childhood skin TB has been reported to be 18.7% of all cases of skin TB in Chandigarh, 20.4% in Varanasi, 24.41% in Chennai and higher prevalence ranging from 31.7–53.9% in a recent case series from Delhi. The difference in local prevalence of TB in certain parts of North India is suggested by a differential accumulation of cases over a period of time [16–19].

Another multi drug resistant skin infectious disease is leprosy. It is a chronic infectious disease caused by*Mycobacterium leprae*, an acid-fast, rod-shaped bacillus and was recognized in the ancient civilizations of China, Egypt and India. The disease mainly affects skin, peripheral nerves, mucosa of the upper respiratory tract and also eyes. In ancient Indian society, individuals suffering from leprosy were alienated because the disease was chronic, contagious, resulted in disfigurement, had no cure at the time, and was associated with sin. CAP could be the next alternative to treat these types of skin disease where antibiotic drugs cannot reach to the epidermis scale. The reactive oxygen species, atomic oxygen, OH^- radicals, free electrons, ions and other excited species are strongly reactive (before recombination) to treat such skin diseases. It is obvious that in many countries in south east Asia (India, Indonesia, Madagascar, Mozambique, Myanmar, Nepal, Nigeria, Philippines, South Sudan, Sri Lanka, Sudan and the United Republic of Tanzania), recalcitrant skin tuberculosis and leprosy is of major concern and poses significant problems especially when multi resistant isolates are involved leading to nearly untreatable infections in the case of resistance to the (few) drugs of choice INH, Ethambutol, Rifampicin and streptomycin.

In addition, NTM cases continue to increase worldwide giving rise to life-threatening infections especially in immune-compromised patients. As nowadays no striking new drug developments could be expected we investigated CAP susceptibility of mycobacteria (MTBC and selected NTM) in vitro to evaluate CAP as an alternative anti-mycobacterial treatment for skin tuberculosis and disinfection tool in hospital hygiene. Besides *M. tuberculosis* we tested selected NTM to cover a large spectrum of clinical mycobacterial involvement, *M. marinum* as typical species causing water born skin tuberculosis with lymph node involvement, *M. avium* as important cross contaminating NTM with relation to animals, drinking water and patients, *M. chelonae* as typical water contaminant and *M. terrae* as reference test germ and rare pathogen of device related nosocomial infections.

[3] MTB: Mycobacterium tuberculosis.

2. Materials and methods

2.1. Plasma sources

2.1.1. Atmospheric pressure plasma jet (APPJ), kINPen 09 (INP Greifswald, Greifswald, Germany)

The schematic setup of the APPJ device used in this study is given in figure 5. For a detailed characterization of the APPJ see [40]. Briefly, in the center of a quartz capillary (inner diameter 1.6 mm), a pin-type electrode (1 mm diameter) is mounted. Argon as the feed gas flows through the capillary (gas flow rate of about 8 L/min). A radio frequency (RF) voltage (1–5 kV, 1.5 MHz) is coupled to the center electrode. The plasma is generated from the top of the center electrode and expands to the surrounding air outside the nozzle.

To avoid killing effects (and skin damage for future application) by constructional reasons the temperature at the tip of the beam with a length of up to 12 mm did not surpass 37 °C as a result of the cooling effect by the argon stream together with the electronic regulation when the device works with 60 V (Fig.6). The tip of the beam is defined as the distal end of the visible pointed discharge. The temperature profile of the flame is shown in figure 6. The Jet can be used in non pulsed and pulsed mode with same physical properties but less heat development.

2.1.2. Dielectric Barrier Discharge (DBD, Cinogy GmbH, Duderstadt, Germany)

The dielectric barrier discharge is generated by the application of high voltage pulses (14 kV) across small gaps with the high voltage electrode being covered by a dielectric barrier made of macor (Fig. 7). The diameter of the glass ceramic electrode measures 20 mm. The pulse repetition rate is adjustable between 100 and 400 Hz which leads to electric powers dissipated in the gas discharge in the range of 167–237 mW. Hereby, transferred electric energy per pulse was measured by Lissajous method and power was calculated according to frequency [47,50].

2.2. Antimicrobial plasma treatment

The following clinical mycobacterial isolates were kindly provided by Sabine Rüsch-Gerdes, German National Reference Center for Mycobacteria, Borstel, Germany and used in the study: *M.tuberculosis*, isoniazid (INH) resistant, *M. avium*, *M. chelonae*, *M. ulcerans*, *M. terrae*, *M. abscessus*, *M. marinum* and *M. africanum*. Suspensions from all isolates were prepared in adequate media and finally quantitatively plated onto Löwenstein-Jensen medium in petri dishes (d = 90 mm) giving growth of nearly confluent colonies after 48 h (fast growing species) up to one week (*M. tuberculosis*). Directly after plating, the medium was manually treated by CAP at a 90° angle over 0, 3, 15, 30 and 60 s. Directly after treatment the plates were incubated aerobically at 36 °C in the dark (except *M.marinum* which was incubated at 30 °C). After the occurrence of visible colonies, the growth-free zones (inhibition zones) were measured and expressed as diameters (d) The details of the test procedure are described in previous publications [7,8,31].

2.3. Statistical analysis

The reduction of the plasma treatment was calculated by comparing the obtained growth-free areas after treatment expressed as diameter of the circular germ-free zone on medium. We used the Mann-Whitney u-test with a significance level of 5%.

3. Results

Both plasma sources caused significant growth-free zones of all mycobacterial isolates on Löwenstein-Jensen medium ($p < 0.01$) already after 3 s CAP treatment. The killing zone diameters increased with increasing treatment time up to 60 s except for MTB which showed