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THE USE OF BACTERICIDAL EFFECTS OF CLUSTER IONS GENERATED BY PLASMAIN MEDICAL BIOTECHNOLOGY

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Introduction

Most of the people spend much time indoors, which makes it an important microenvironment in terms of air pollution. Due to numerous studies, the understanding rises that the indoor air is highly complex, somewhat unpredictable and potentially dangerous. Together with Volatile Organic Compounds (VOCs), the health implications of indoor microbial contaminants have become an issue of increasing concern in recent years (1-6). It is recognized that microorganisms constantly present in indoor environment can cause a spectrum of illnesses in humans, ranging from allergic illnesses to invasive disease (7).

If associated with microbial contamination of buildings, diseases are considered "building-related illnesses" (BRI) (7,8,9). The role of microbes is not well defined in "sick building syndrome" (SBS), but it is this syndrome that most frequently prompts building investigations. Many air pollutants access the body via inhalation of indoor air because of both the percentage of lifetime spent indoors and higher indoor pollution levels (1-3).

House dust is a mixture of different organic and inorganic constituents, including human and animal dander, feathers, textile fibers, food particles, kapok, particles of mites and other arthropods, pollen, algae, fungal spores and mycelia, bacteria, and soil particles. Many of these have been implicated in the etiology of hypersensitivity pneumonia, allergic rhinites, and some types of asthma.

Infectious illnesses, such as influenza, measles and chicken pox are transmitted through air. The recent SARS outbreak has shown once again that infectious diseases that are possibly spread by the respiratory route must be taken very seriously. Here, it is worthy to note that only 10% of all colds are caught out-

doors. Besides those origins of diseases there are molds and mildews releasing disease-causing toxins. Very young and elderly people in particular belong to the high-risk group (3-6).

The most of conventional methods of air purification use the power of a fan to draw in air and pass it through a filter. The problem of bacterial contamination of inner parts of such a type of air conditioners in some cases draws attention towards alternative air-cleaning systems. Some manufacturers offer to use the ozone's bactericidal and deodorizing effects, but the wide spreading of such systems is restricted by the fact that toxic effects of ozone in respect of human beings are well known. In 2000 Sharp Inc. introduced "Plasma Cluster Ions (PCI)" air purification technology, which uses plasma discharge to generate cluster ions (10-14). This technology has been developed for those customers that are conscious about health and hygiene.

The new plasma cluster method releases positive and negative ions, into the air, which are able to decompose and deactivate harmful airborne substances by chemical reactions (10-15). Because cluster ions consist of positive and negative ions that normally exist in the natural world, they are completely harmless and safe. The amount of ozone generated by cluster ions is less than 0.01 ppm, which is significantly less than the 0.05-ppm standard for industrial operations and consumer electronics. This amount, thus, has no adverse effect whatsoever on the human body.

A lot of phenomenological tests of the PCI air purification technology on microbial cells have been conducted. And, in most cases; it has been shown that there exists an well-shaped anti-microbial effect. However, the positive and negative results still have not been adequately interpreted from the biological point of view, as the particular chemical mechanisms of PCI actions are still unknown. It is suggested that ions cluster in contact with micro particles (such as bacteria and odour-causing molecules) chemically react with them through free-radical-mediated mechanism leading to the destruction and modification of those micro particles. The other mechanism possible mechanism is that air-ion particles are attracted to pollutant particles, and as they join, they become heavy and cannot stay airborne.

In our experiments, we focused on some principal aspects of plasma-generated ions application – time-dependency and irreversibility of bactericidal action, spatial and kinetic characteristics of emitted cluster particles, their chemical targets in the microbial cells.

Materials and methods

Microbial strains and cultivation

The main criteria in microorganisms selection were ubiquity and stability in natural and artificial environments (indoor, outdoor), non-pathogenicity, variability in biochemical properties (structure and metabolism).

We used the strains *Staphylococcus chromogenes* (Devriese et al. 1978) ATCC 43764, *Enterococcus malodoratus* (ex Pette 1955) ATCC 43197, *Sarcina flava (Micrococcus luteus)* DSM No. 20030, ATCC 4698, *Micrococcus roseus (Kocuria rosea)* DSM No. 20447 ATCC 186. These bacteria were cultivated at 37°C on Casein-peptone Soymeal-peptone Agar (CASO agar) (Merck) and in CASO Broth (Merck). Conical flasks 250ml with CASO broth were used in long-time cultivation.

Evaluation of bacterial cells viability

Two main methods have been used to determine the bacterial viability - colony-forming units (CFU) counting and differential fluorescent staining.

A common criterion for bacterial viability is the ability of a bacterium to reproduce in suitable nutrient medium. In regular CFU experiments, 30 µl volume of bacterial suspension in PBS buffer (pH7.4) after serial dilutions was poured on agar medium and was spread by glass spatel.

The number of survived bacteria was estimated as the number of CFU on each pour plate. The number of colonies on the plates was counted after 72 hours of incubation. (The number of colonies was between 20 and 300 colonies). Any plate which had more than 300 colonies was designated as "too numerous to count" (TNTC) and discarded. Plates with fewer than 20 colonies were discarded too as statistically unacceptable.

It should to be emphasized that methods relying on metabolic or growth characteristics are not always adequate because injured bacteria may sometimes form colonies. Conversely, some bacteria with intact membranes may be unable to reproduce in nutrient. For that reason, Bacterial Viability Kits (Molecular Probes) has been used as an additional method for bacterial viability estimation. LIVE/DEAD® BacLightTM is a novel two-colors fluorescence assay of bacterial viability that has proven useful for a diverse array of bacterial genera.

This method utilises mixtures of our SYTO® 9 green-fluorescent nucleic acid stain and the red-fluorescent nucleic acid stain, propidium iodide. These stains differ both in their spectral characteristics and in their ability to penetrate healthy bacterial cells. When used alone, the SYTO-9 stain generally labels all bacteria in a population — those with intact membranes and those with damaged membranes. In contrast, propidium iodide penetrates only bacteria with damaged membranes, causing a reduction in the SYTO-9 stain fluorescence when both dyes are present. Thus, with an appropriate mixture of the SYTO-9 and propidium iodide stains, bacteria with intact cell membranes stain fluorescent green, whereas bacteria with damaged membranes stain fluorescent red. The excitation with laser 480/490 nm has been used for visualisation.

Specifically, 50 µl of bacterial suspension in distilled water have been applied to microscope glass slide, dried, and, after pci exposure for a certain period of time, a 20 µl of dye mixture were added (16). Than the dye mixture was trapped between a slide and an 18-mm square cover slip, incubated at room temperature in the dark for 15 minutes and was observed in a fluorescence microscope karl zeiss equipped with specific optical filters.

Plasma cluster ion generation and detection

Cluster air-ions were generated by means of Plasma cluster Ion Generator kindly provided by SHARP Co. Ion detector „IST-801A" and Monitoring software Lab View module Run-Time Engine 6.02 (National Instruments) has been used for Measurements of air-ions concentration in the air. This hardware-software combination allows selective real-time detection of either negative or positive ions in the air.

Biochemical examination of chemical nature of plasma-generated ions

For superoxide detection, we used ability of 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt (WST-1) to transform to purple-colored WST-1- formazan form under presence of O2- free radicals. During the experiment, 5x100 aliquots 0,01M solution of WST-1 in distilled water were treated by cluster ions for 2 hours. The presence of WST-1- formazan was estimated both visually (by the change of colour) and photometrically at 438nm (formazan peak).

Hydrogen peroxide detection was based on the fact that in the presence of horseradish peroxidase (HRP), the Amplex Red reagent (10-acetyl-3,7-dihydroxyphenoxazine) reacts with a 1:1 stoichiometry with H2O2 to produce highly fluorescent resorufin (7-Hydroxy-3H-phenoxazin-3-on). To check the presence of hydrogen peroxide, 5x100 aliquots 0,01M solution of Amplex Red in phosphate saline buffer were treated by cluster ions for 2 hours. The presence of resorufin was estimated after adding 1 mM of peroxidase, both visually (by the change of colour) and photometrically at 563 nm.

In the case hydroxyl radical detection, the experimental approach has taken into account the ability of hydroxyl radical to facilitate the conversion of hydrogen peroxide to superoxide radical. The colourless mixture of 0,01M WST-1 and 2% hydrogen peroxide has been exposed to cluster air ions for 2 hours and a appearance of colour due to formazan formation has been observed both by naked eye and photometrically (see above).

Examination of time-dependency of PCI action.

Using sterile plastic tips, decimal dilutions of microbial suspension were prepared. 20 μl volume of final dilution was pipeted into appropriately marked Petri dishes with CASO agar. Each variant of PCI treatment was carried out in triplicate. Variants differed only in duration of exposure to PCI, usually, 0, 30, 60, 120, 180, 240 and 480 minutes, respectively. PCI treatment was carried out by placing opened Petri dishes into acryl experimental chamber, onto horizontal surface (distance from PCI generator 10±0,5 cm). After all variants being treated, all Petri dishes were placed in thermostat. After 48 hours colonies on agar were counted .

Irreversibility of inhibitory effect of plasma cluster air-ions and the influence of culture medium treatment alone.

Three different methods have been used to examine whether PCI action is irreversible or not.

1. After appropriate PCI treatment (see above), experimental fraction of Petri dishes were stored at 4°C (when destructive processes are lingering) for 3 days and then viability has been checked. The control fraction has been checked according to a standard procedure.

2. Incubation of the Petri dishes for longer period (2 weeks) in thermostat. This approach is based on the assumption that some colonies could appear after 48-hours time. The bacteria were cultivated on agar plates at 37°C for 14 days and the plates were visually inspected for bacterial growth.

3. Passage of untreated microbial cells onto the PCI-treated agar culture medium (to detect possible influence from PCI treated culture medium)

Determination of spatial characteristics of PCI emission

Since the direct spatial characterization of PCI propagation properties is difficult due to relative size and shape of experimental chamber and used portable ion detector, the indirect approaches have been applied. Specifically, the PCI spatial concentration has been measured both by estimation of bacterial growth inhibition rate and by so called "substrate film" method.

In inhibition rate calculation experiments, Petri dishes, after standard procedure of inoculation with bacteria, were attached to every inner side of experimental chamber. Thus, almost all inner surfaces of chamber could be regarded as one big test plate on the analogy of experiments described above. The plasma cluster ion generator was switched on to 1 hour and than all Petri dishes (including the control ones, incubated in control chamber) were placed in incubator for 48 hours for posterior counting of colonies.

In "substrate film" method, all walls of experimental chamber were covered with a cellophane film (Roth, Germany) priory uniformly wetted with substrate solution (reaction mixture of 0,01M WST-1 and 2% hydrogen peroxide in phosphate buffer pH 7.4). In the course of time, the PCI-induced formation of WST-1 formazan was detected by purple color appearance. On the ground of color intensity distribution, the spatial relative concentration of PCI has been evaluated.

In a separate series of experiments (spatial obstacles experiments), the ability of plasma generated ions to penetrate the almost isolated locations has been studied. Such an information could allow to speculate about the character (length, shape) of trajectories of plasma cluster ions inside the chamber. So, after a standard procedure of bacterial inoculation, half of the opened Petri dish in the chamber has been covered (screened) by polypropylene film. This film allowed creating steric difficulties on the way of particles to particular part of the Petri dish. After PCI exposure for 1 hour, the film has been removed and closed Petri dish has been placed into incubator. The relative number of colonies in both halves of Petri dish has been calculated after 48 hours of incubation.

Analysis of bacterial proteins.

The changes in protein composition of microbial cells have been detected in SDS PAGE. Cells were harvested, washed, treated (as a dense suspension) with cluster air ions, than subjected to 3 freeze-thaw cycles in extraction buffer (Tris-HCl pH7, 2%SDS, 5mM EDTA, 1mM PMSF). After centrifugation at 11 000g, SDS-PAGE of supernatant has been running. using 10% (wt/vol) acrylamide gels.

Proteins were visualized and quantitatively characterized by staining the gels with Coomassie brilliant blue R-250 (17). Silver staining was carried out according to a standard procedure. After gel drying, scanning and digital processing were carried out. Gels were scanned with a personal densitometer Kodak and molecular weights were estimated by using the DIAdem software (version 8,0; National Instruments) with molecular weight markers as standards.

Statistical analysis of data

Mathematical apparatus for the case of normal distribution was used in analysis of all data obtained. After several experimental iterations, a mean, a standard deviation and a standard error of the difference be-

tween the means have been calculated. Conventional t –test has been used to estimate the significance of differences between variants.

Error bars showing the standard error of the mean (SEM) have been used to aid interpretation of the data in the graphs. Correlation coefficients and linear regression trend lines were calculated and plotted using Microsoft® Excel 2000, normalization of data was carried out in DIAdem Software (National Instruments).

Results and discussion

Ion emission measurement

Previous studies have demonstrated that plasma cluster generator emit both negatively and positively charged ions with variable charge/mass ration, and, respectively, with different motility. In this study, the emission of negatively and positively charged cluster ions by given generator in the course of time has been examined. .

The PCI emission has a very high local fluctuations as times goes by, but at the given longer period of time the average ion concentration in the chamber air was nearly the same. The peculiarities of the positive and negative ion emission under different conditions (air temperature, humidity, when the chamber was closed tightly and then a small gap existed) has been examined too, with the aim in view to obtain more adequate data in further experiments with involvement of bacteria.

Observable damage of colonies

After treatment with plasma-generated cluster ions for 8 hours an profound changes in colony morphology can be observed. Colonies are not regular round shape, not smooth surface, a lot of radial crackles appeared.

It is worthy to emphasize, nevertheless, that no visible micro-morphological damages (size, shape, staining properties) have been detected on the level of individual cells, when usual staining protocols were applied (gram staining, phase contrast, FITC-staining, etc.).

Time-dependent action

It is important to note that most experiments were carried out using bacterial cells attached to surfaces and not aerosolised. Such an approach is stipulated by the well-recognized fact of higher physical and chemical resistance of microbial cells attached to solid surfaces comparing to the free ones. Partially, that can be explained in terms steric conditions of reaction. The other reason is simplicity in performance and interpretation of results, because the possible participation of aggregation and sedimentation mechanisms can be avoided such a way.

The effect of cluster ions on growing cells of bacteria, compared with the control: the exposure of growing cells to PCI caused significant reductions in the numbers of viable cells after different periods of incubation. For Staphylococcus and Enterococcus, analysis of variance showed a significant difference ($P < 0.01$) in bacterial number with respect to the control after 30 min of treatment with PCI. Three hours of incubation resulted in almost full inhibition of the growth.

It is worthy to note that the inhibition rate (time-kill) curves are almost linear in case of natural logarithm scale been used. That allow us suggest the prevalence of single-hit single target mechanism in PCI killing action. In that case the reduction in microbial population can be described by the first-order reaction model like

$$S = \exp(-kt)$$

In this equation S represents the fraction of the original population that survives exposure at time t. The rate constant k can be determined experimentally for particular kind of bacteria.

D37 dose, when the ratio between amounts of survived cells and total amount of cells is equal exp(-1) was reached by

Staphylococcus chromogenes in 26 min

Enterococcus malodoratus in 45 min

Sarcina flava in 194 min

Micrococcus roseus in 129 min

Noteworthy, both Sarcina and Micrococcus contain a lot of carotenoid pigments and the protective properties of pigment extracts have been demonstrated in separate series of experiments (no published data). Hence, the higher resistance observed in our trial could at least in part be explained by the higher carotenoid content of these strains.

Irreversibility exper:ments

The irreversibility of effect may be important during the discussion about the relative prevalence of either bacteriostatic or bactericidal mechanisms in antimicrobial action of plasma-generated air-ions. In irreversibility experiments, irrespective of the particular method used, it has been shown that the effect of PCI on microbial cells is irreversible and does not mediated by changes in nutrition medium chemical degradation. Obviously, that means an irreversible damage of some cellular components indispensable to life.

Spatial distribution of cluster ions, life time, propagation

It has been shown before that the average PCI life span is about sec and it was supposed that most of air-ions will not continue their movement after collision with surfaces. The domination of such non-resilient collisions has been demonstrated in our experiments with partial, not tight covering (screening) of nutrition agar surface from emitted cluster ions. These experiments have clearly shown that the most of PCI propagate via straight trajectory till they meet some surface.

According to the data obtained (figure) , the most of the PCIs are emitted towards the front and front-downward directions. Thus, it can be regarded as a one more evidence that they are pretty heavy ion clusters with initial momentum of movement and a relatively short lifetime.

Surface-induced chemical reactions of plasma-generated ions

To check whether free radical reactions are involved in PCI action and the dominating type of free radicals, the specific enzyme systems and colour-changing substrates may be used. The significant rate oh hydroxyl radical-specific reactions. Has been detected in our experiments. These results indicate also that there are less or even undetectable amounts of both superoxide radical and hydrogen peroxide.

Quantitative measurements of relative free radical concentration were not be carried out because of some methodological difficulties.

Influence of PCI on bacterial proteins

According to a literature data, the reaction rates for hydroxyl radicals is relatively low in respect of carbohydrates and nucleic acids and the highest for proteins and for compounds having the conjugated double bounds. Cellular proteins of Enterococcus malodoratus separated by SDS-PAGE, are shown on the Figure 4 for different time of PCI treatment.

The electrophoretic pattern for proteins from the different exposure time variants was clearly different, especially in the range 34 and 93 kDa.

For all variants, PCI effect was characterized by the aggregation and splitting events in almost all protein bands. Coomassie blue and silver-stained gels showed also that the relative amount of these proteins changed sharply with increasing exposure time.

Results of this study support the hypothesis that antibacterial properties of plasma-generated cluster ions can be explained in terms of "in situ" free-radical formation.

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