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Spraying of Cell Colloids in Medical Atomizers

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Aerosols are often used as vehicles of medicines and their most important application is related to inhalation of anti-asthmatic or anti-inflammatory drugs. Aerosols are also convenient in topical applications, i.e. applied on skin or mucous membranes (e.g. of nasal or oral cavity). Recently, it has been shown that use of a therapeutic bio-aerosol can be beneficial in the speeding up of healing processes of the skin. In this context, cell suspensions are considered as the material for which the suitable spraying method must be established in order to assure that hydrodynamic stress related to liquid atomization will not destroy the living cells. The current work is focused on testing of the influence of selected spraying techniques on the integrity and the survival of different types of cells in the atomized bio-colloid. Medical nebulizers and similar spraying devices have been characterized in respect of the size distribution of emitted droplets, and used to aerosolize selected (i.e. model) cell colloids: suspensions of yeast, bacteria and murine fibroblasts. Cell viability after spraying has been determined via direct microscopic observations and specific microbiological assays. The results shows that droplets generated from the commonly used medical inhalers (nebulizers) are too fine to contain whole living cells (median diameter of drops is around 5 μm). Because the generation of small droplets requires high hydrodynamic stresses, viability of cells in the atomized suspension is strongly reduced even if cells are mechanically resistant and small in size. Tested nasal atomizer (spray pump) and Microsprayer devices produce larger droplets (median diameter of drops ≈ 50-80 m) which can contain integral fibroblast cells. Owing to lower shear stresses during atomization in these two devices, the aerosolized cells remain viable and capable of proliferation. The studies allow to conclude that only selected techniques can be effective in the atomization of bio-colloid for medical applications. Moreover, the atomization method must be accompanied by proper techniques of aerosol transfer to the bronchial tree which will prevent droplets from deposition in the upper airways. Technical concepts of such cell delivery are proposed. This paper is an extended version of our recent work published in Chemical Engineering Transactions (Sosnowski et al., 2013).

1. Introduction

Atomization of bio-colloids, i.e. suspensions which contain living cells has a potential application in regenerative therapies. As an example, autologous cells administered topically in form of aerosol have been successfully used in healing skin burns and scars (Gravante et al. 2007, Gerlach et al, 2011). There is also a chance of using atomized cells to accelerate repair of the lungs which can be seriously damaged due to inhalation of hot or aggressive gases and vapors (Angelini et al, 2013). The natural way of delivery of therapeutic agents to the lungs is conversion to the form of aerosol which may be naturally inhaled and deposited inside the respiratory system. In this way the medicine can reach even distal parts of the bronchial tree and is fairly uniformly spread in the whole breathing organ. Typically, drugs for asthma are targeted to the lungs as aerosol produced in different types of medical inhalers: pressurized metered dose inhalers (pMDIs), dry powder inhalers (DPIs) or liquid atomizers, also known as nebulizers (Newman, 2009; Sosnowski, 2012).
In this work, which is an extended version of our recent paper published in Chemical Engineering Transactions (Sosnowski et al., 2013), we consider the application of aerosol as a carrying medium for suspensions of cells

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which might be delivered to the respiratory tract in order to regenerate damaged lung tissue. We focus on the applicability of selected medical atomizers for spraying such bio-colloids. The results of this basic research should help in the rational selection of atomizing devices for cell delivery in the regenerative medicine of the lungs (i.e. by decellurization followed by cell seeding - Petersen et al., 2011) but also of other organs (e.g. delivery to skin and mucous membranes reachable by aerosol).

2. Materials and methods

2.1 Biological material

Three types of model cells were used in the studies:
a) yeast cells: *Saccharomyces cerevisiae* (Lessafre, Poland),
b) bacterial cells: *Lactobacillus casei* (Lek-AM, Poland),
c) animal (mammalian) cells: mouse fibroblasts, line L929 (European Collection of Cell Cultures, UK).
As observed by optical microscope, the cells were characterized by different size - yeast: 5-10 μm, bacteria: 2-4 μm, fibroblasts: 30-130 μm. The appropriate aqueous/buffer suspension of each type of cells was prepared just before atomization studies. Yeast and bacteria were studied in non-sterile conditions, while in all investigations with fibroblasts sterile conditions and media were applied.

2.2 Atomizing devices

Five types of medical atomizers (Figure 1) which operate on different spraying principles were used:
a) pneumatic nebulizer, PN – model RF6 (FlaemNuova, Italy), supplied with the compressed air delivered from MP1 medical compressor (Medbryt, Poland),
b) ultrasonic nebulizer, US - model Thomex (Medbryt, Poland),
c) vibrating-mesh nebulizer, VM - model AeroNeb (Aerogen Inc., Ireland),
d) Microsprayer® Aerosolizer, MSA - model IA-1B (Penn-Century Inc., Wyndmor, PA, USA),
e) nasal atomizer, NA (i.e. spray pump - Coster, Italy).

Pneumatic, ultrasonic and vibrating-mesh nebulizers (PN, US and VM) were electrically driven while two mechanical atomizers (MSA and NA) were operated manually.

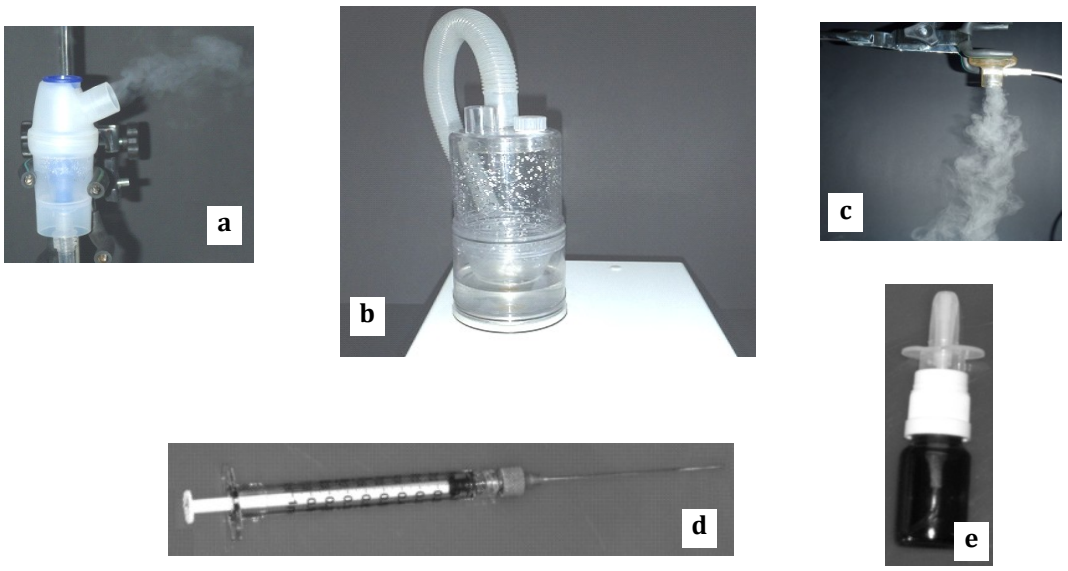

Figure 1: Atomizing devices used in the studies: a – pneumatic nebulizer RF6 (FlaemNuova), b – ultrasonic nebulizer Thomex (Medbryt), c – vibrating mesh nebulizer AeroNeb (Aerogen), d – Microsprayer Aerosolizer (Penn-Century), e – nasal atomizer (Coster).

2.3 Droplet size distribution determination

Size distribution of droplets produced by the atomizing devices was measured with Spraytec diffraction aerosol spectrometer (Malvern Instruments, UK – Figure 2) characterized by the detection size range of 0.1 - 2500 μm. In contrast to other optical methods, the diffraction spectrometry allows for on-line analysis of the raw cloud emitted from any aerosol source without the need of aerosol dilution. The device was operated in the "open-bench" configuration with 1 kHz sampling rate with the automatic trigger of the measurement. The results collected during 500 ms sampling were time-averaged.

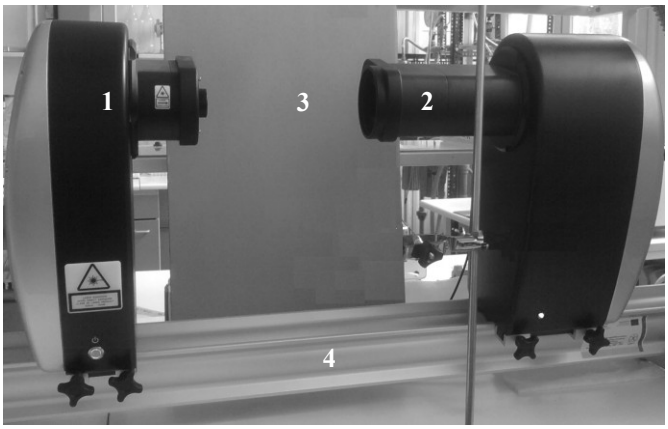

Figure 2: Diffraction aerosol spectrometer Spraytec (Malvern Instruments, UK) used for the measurement of droplet size distribution: 1 – laser emitter, 2 – laser detector, 3 – measuring zone, 4 – optical bench.

2.4 Procedures for cell viability and integrity studies

Cell survival during atomization in the ultrasonic device was evaluated only for the aerosol which was formed and recycled inside the nebulizing vessel (Figure 1b) because collection of the emitted droplets in amounts needed for cell counting was not possible. For pneumatic and vibrating-mesh atomizers cell viability could be determined both in the atomized liquid circulating inside the nebulizing vessel and in the liquid collected after capturing of emitted aerosol. For MSA and NA devices the cell survival was analyzed solely in the liquid emitted as aerosol. Depending on the cells type used several different assays were applied:

a) for yeast cells – the vital staining (methylene blue 0.01%) and microscopic evaluation using Thoma counting chamber,
b) for bacteria – the microscopic evaluation of cell integrity observed in Thoma chamber,
c) for fibroblasts – three assays were used: (i) vital staining with trypan blue (0.04 %) after cells trypsinisation, (ii) fluorescence test ("Life/Dead"), and (iii) MTT colorimetric assay after 48 hr incubation of cells at 37°C.

To evaluate and compare cell viability, the relative number of viable cells, *RVC*, was defined:

$$RVC = \frac{N}{N_0} \cdot 100\% \quad (1)$$

where: N denotes the fraction of viable cells (by number) in the given sample, N_0 – the fraction of viable cells in the initial sample (i.e. the control sample without atomization).

In case of bacteria cells the viability could not be evaluated by vital staining with methylene blue, therefore the relative amount of intact cells, *RIC,* was defined for the sake of sample comparison:

$$RIC = \frac{K}{K_0} \cdot 100\% \quad (2)$$

where: K denotes the number of intact (whole) cells visible in the field of view counted in Thoma chamber, K_0 – the number of whole cells visible in the same view-field in the control sample (i.e. without atomization).

All cell countings and viability assays were done in many repetitions to obtain the reliable average and standard deviation data.

3. Results

3.1 Droplet size distribution

Droplet size distribution in the aerosol emitted from different atomizers is depicted in Figure 3 as a volumetric (mass) frequencies.

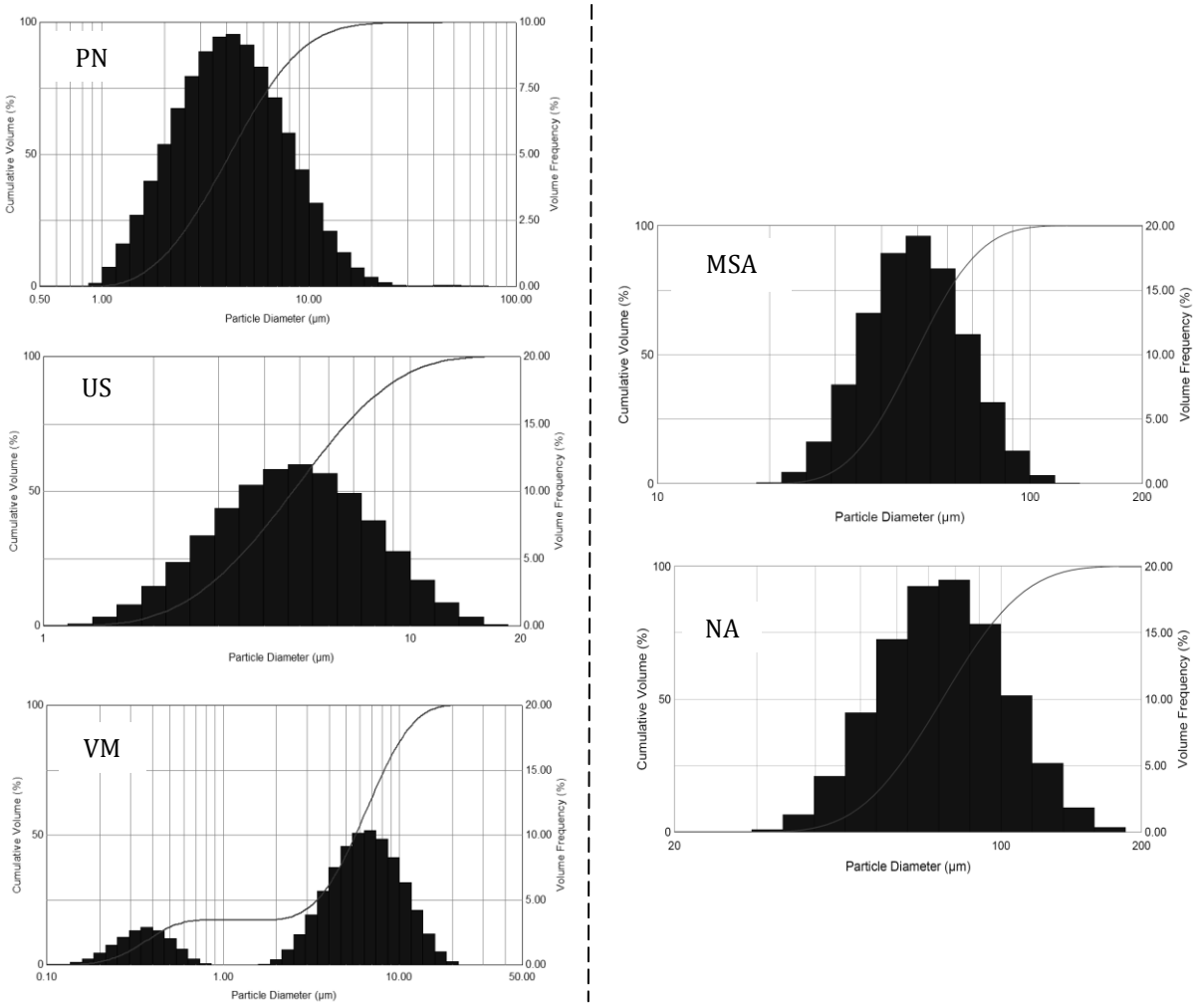

Figure 3: Size distribution of droplets produced by all tested atomizing devices: left panel – nebulizers, right panel – mechanical sprayers (designations as defined in chapter 2.2.)

Based on the data presented in Figure 3 it is highly improbable that large cells (such as fibroblasts) could be sprayed without substantial destruction in the commonly used medical nebulizers (PN, US, VM) for which all emitted droplets are smaller than 20 µm. Therefore, in the further cell survival studies these three nebulizers were used only for yeast and bacteria (cells with the average size below 10 µm) in order to test if the hydrodynamic stresses associated with these spraying techniques can be considered as safe for cells. In case if the spraying in nebulizers is not damaging to these cells, the application of such atomization devices to generate cell aerosol for regenerative purposes will remain potentially applicable if sufficiently small cells are considered (e.g. stem cells - Zouani et al., 2012). However, it must be kept in mind that the mechanical resistance of cell wall of yeast and

bacteria is higher that of the cell membrane of animal cells (e.g. Touhami al, 2003), so the separate tests on small stem cells would be still necessary in the future.
Two mechanical atomizing techniques (MSA, NA) allow to obtain significantly larger droplets (30-150 μm), so they seem much more suitable for spraying of bio-colloids containing larger and more sensitive animal cells. Production of droplets with this size requires significantly weaker hydrodynamic stresses during atomization process. As the energy converted to disperse the unit volume of the liquid is proportional to the newly created surface area in the system, it can be estimated that generation of droplets with the average size of 50 μm will require average stresses which are 25 times less than the ones needed to produce 10 μm droplets. On the other hand, the important practical problem in the aerosol usefulness for lung regeneration lies in the limited suitability of coarse droplets for targeting the lower respiratory airways. Typically, droplets larger than 10 μm have a very low chance to penetrate beyond the throat and trachea during normal inhalation (Sosnowski et al., 2006). Our recent studies indicate, however, that direct introduction of large droplets to the trachea may improve their delivery to the bronchial tree. We will address this issue in the "Discussion and Conclusions" section.

3.2 Survival of yeast and bacterial cells during atomization in medical nebulizers

Cell survival during pneumatic atomization (PN)

The relative fractions of viable yeast cells (RVC) in the liquid which contains the aerosol recycled inside the pneumatic nebulizer, and in the liquid which was emitted as aerosol droplets from this device are depicted in Figure 4. It is seen that yeasts are generally resistant to the mechanical stresses associated with liquid atomization in PN device. After 6-8 minutes of spraying, 90% of viable cells loaded into the atomizer are still alive (Figure 4a), and this rate of survival is only slightly lower (~80%) for cells collected from the emitted aerosol (Figure 4b). Survival of the bacteria in the same nebulizer is illustrated in Figure 5, and the result indicate noticeably lower fractions of intact cells: only 20% inside the vessel (Figure 5a) and practically zero in the aerosol after 6 minutes of atomization (Figure 5b). Due to significant time-dependent destruction of bacterial cells and small size of produced droplets, this atomizer was excluded from further tests with fibroblast cells.

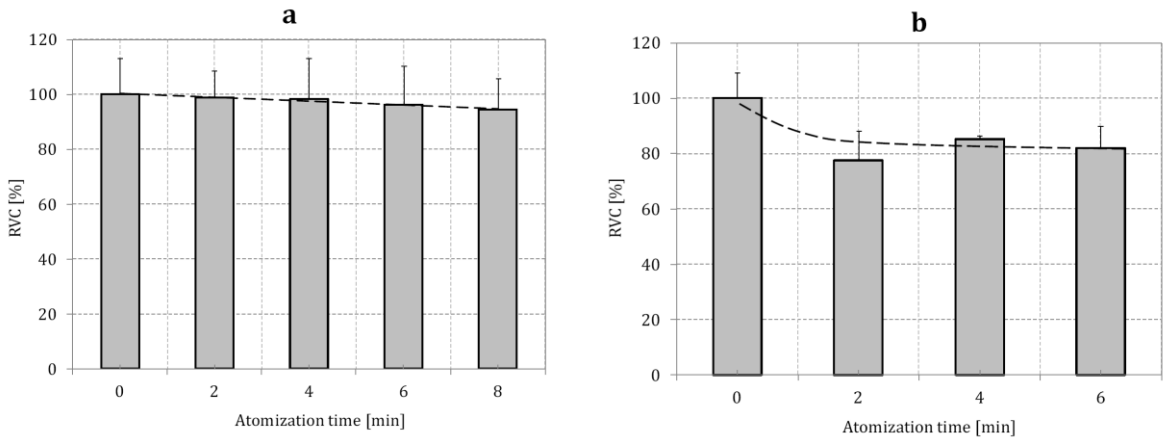

Figure 4: Survival of yeast cells during pneumatic atomization (PN): a – viable cells in the liquid inside the atomizing vessel, b – viable cells in the liquid emitted from the nebulizer as droplets.

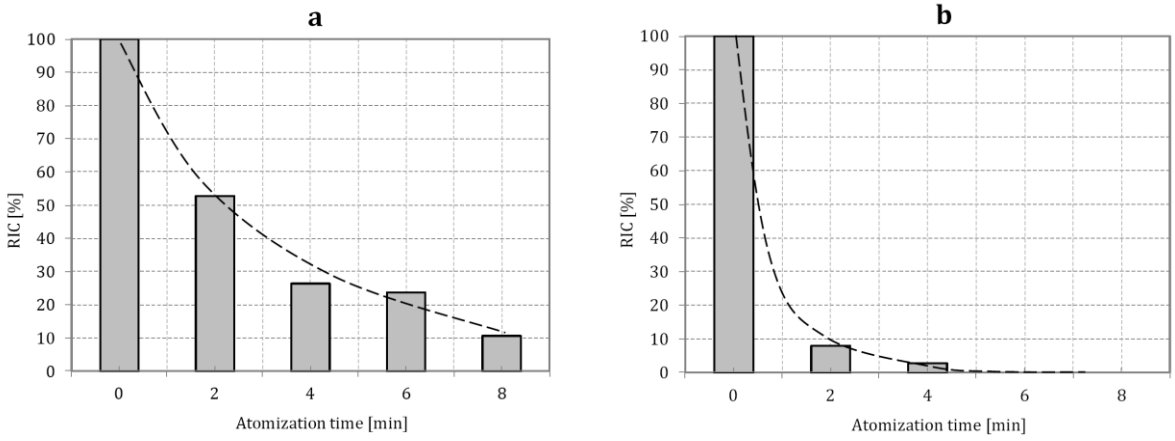

Figure 5: Survival of bacterial cells during pneumatic atomization (PN): a – intact cells in the liquid inside the atomizing vessel, b – intact cells in the liquid emitted from the nebulizer as droplets.

Cell survival during ultrasonic atomization (US)

The data on cell viability during ultrasonic atomization are shown in Figure 6. Both yeasts and bacteria are noticeably destroyed during the process due to the stresses generated by ultrasounds. The transient increase in number of viable yeast cells after 4 minutes of atomization (Figure 6a) may be related to the stress-induced reproduction. Taking into account also the size distribution of droplets produced in this device (Figure 3), it is evident that US nebulizer cannot be used for spraying animal cell which are larger and more sensitive.

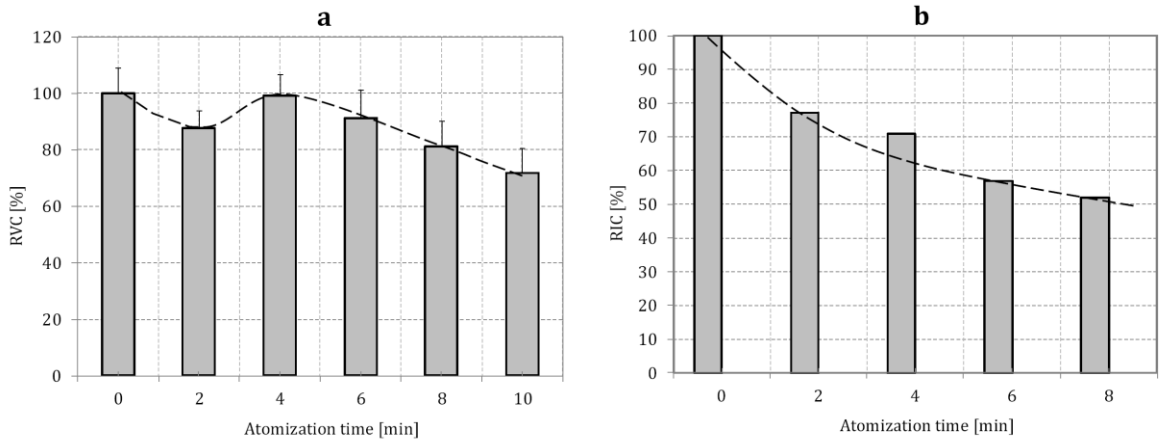

Figure 6: Survival of cells during ultrasonic atomization (US). The cells were collected from the aerosol recirculated inside the nebulizing vessel: a - yeast, b - bacteria.

Cell survival during atomization in the vibrating mesh nebulizer (VM)

Results of cell viability during atomization in VM nebulizer are shown in Figures 7 (for the yeasts) and 8 (for the bacteria). In both cases a significant decrease of the fraction of viable cells is observed as a function of spraying time. On the same rationale as US nebulizer, the VM device is considered inappropriate for atomization of animal cells.

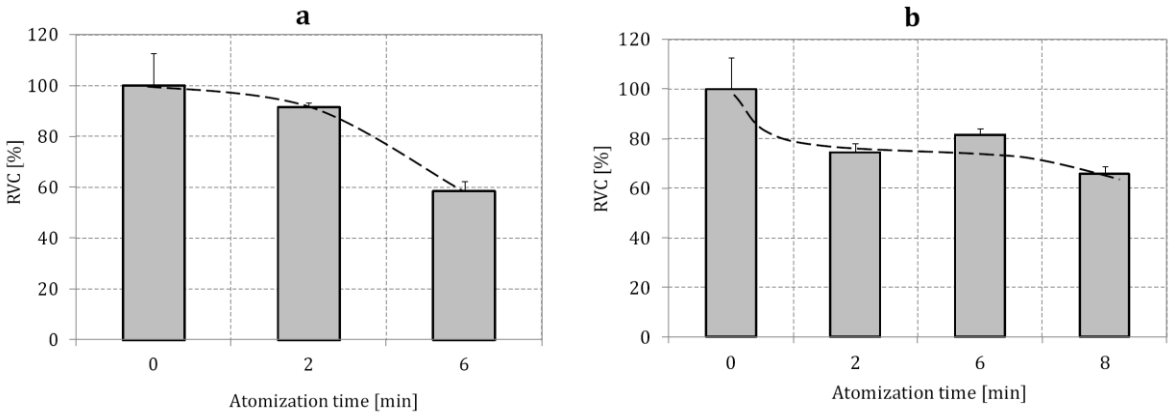

Figure 7: Survival of yeast cells during VM atomization: a – viable cells in the liquid inside the vessel, b – viable cells in the liquid emitted from the nebulizer as droplets.

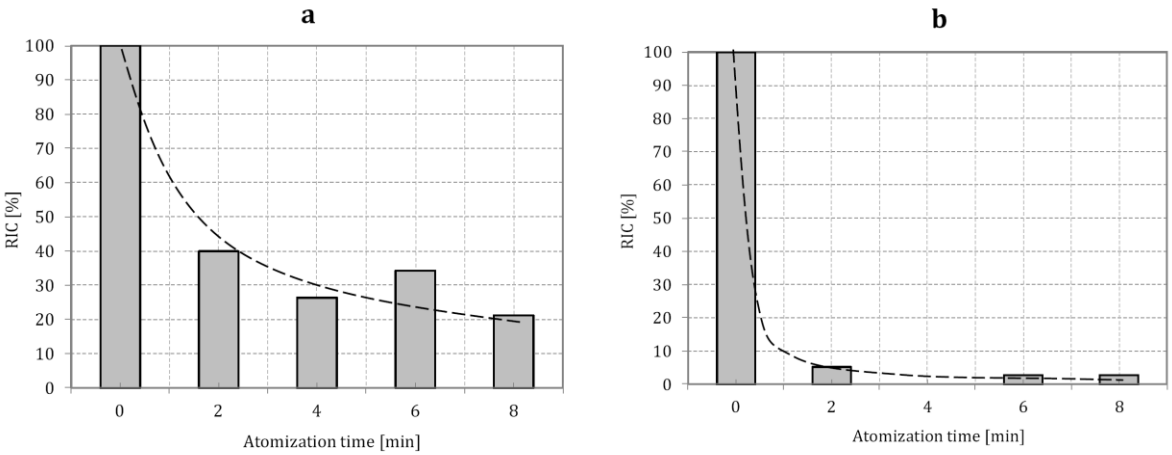

Figure 8: Survival of bacterial cells during VM atomization: a – intact cells in the liquid inside the vessel, b – intact cells in the liquid emitted from the nebulizer as droplets.

3.3. Survival and activity of fibroblasts after spraying in MSA and NA devices

Due to a larger size of fibroblast cells, all tested nebulizing techniques (PN, US, VM) had to be excluded as they generate too fine droplets which cannot contain the entire cell. Therefore only two other atomization methods (MSA and NA) have been tested in fibroblasts spraying. As stated in section 2, the survival rate for these animal

cells was determined using more complicated assays which required sterile conditions. Figure 9 summarizes the results of three different tests. In all assays the survival rate of fibroblast cells was very high (above 94%) indicating that both MSA and NA atomization techniques are suitable for spraying viable animal cells. The enzymatic MTT assay of fibroblasts sprayed in the nasal atomizer (NA) which was done after 48 h of cells incubation indicates a higher loss of cell activity (65% of the control) than in case of spraying in MSA, suggesting that atomization in the nasal pump is more destructive and can lead to death of cells over time.

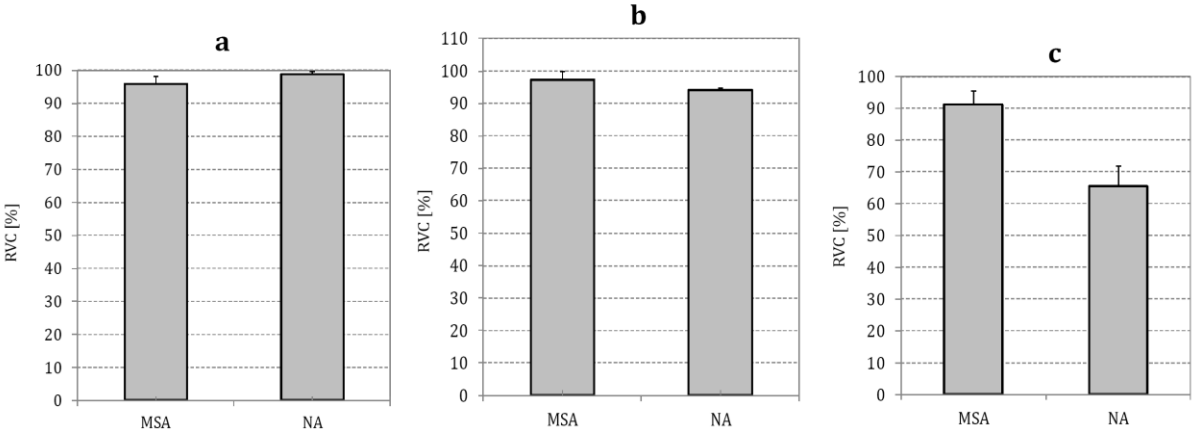

Figure 9: Survival of fibroblasts atomized in MA and NA devices determined by different assays: a) "Life-Dead" fluorescence test, b) Trypan blue staining, c) MTT test (48 h after spraying).

Our new results (Sosnowski and Tomecka, 2013) as well as the recently published studies (Kardia et al., 2013) indicated that fibroblasts atomized in MSA device are capable of effective adhesion and proliferation, eventually forming the confluent cell layer, Figure 10. These data confirm that animal cells atomized in the Microsprayer device preserve their vital functions.

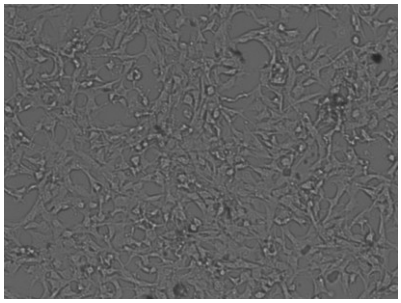

Figure 10. Confluent layer of fibroblasts sprayed from MSA device after 72 hrs of incubation (Sosnowski and Tomecka, 2013).

4. Discussion and conclusions

The presented experimental results indicate that nebulization techniques which are typically used to atomize drugs for inhalation are not suitable for spraying of colloids of animal cells. The average size of droplets produced in these devices (in the order of 5 μm) suggests that single droplet cannot contain a whole cell so it is not possible to transfer bio-colloid to the aerosol phase without mechanical inactivation of the cells. In addition, hydrodynamic stresses associated with generation of such small droplets are very high, therefore even small cells having a more

resistant wall (e.g. bacteria) are disintegrated. This is an important result from practical viewpoint of inhalation therapy since it demonstrates that microbial cells (also potential pathogens) are mostly killed during nebulization. It follows that this should reduce the problem of non-sterility of the nebulizers during their therapeutic use.
In case of selected animal cells, literature data indicate their considerable deformability what allows them to avoid damage under stress (Suchecka et al., 2005). Nevertheless, results of the current study exclude the possibility of animal cells survival following the regular spraying in the nebulizers due to the small size of emitted droplets. Two other atomization devices studied (Microsprayer Aerosolizer and the nasal atomizer) are expected to generate lower hydrodynamic stresses as they produce noticeably larger droplets. The results confirm that these devices efficiently atomize the bio-colloid of fibroblasts without significant damage of cells. We should note, however, that large droplets are virtually inapplicable as vehicles of cells targeted to the lower airways because they will be captured during normal breathing on the surface of the mouth-and-throat region due to inertial impaction (Sosnowski et al., 2006). Nevertheless, some possibilities of delivering aerosolized cells to the bronchial tree still can be proposed. The first concept relies on the intra-tracheal atomization of the bio-colloid using an extended delivery hose with the spraying nozzle mounted at the end. The second idea assumes that aerosol formed as large droplets can be a transferred via the endotracheal tube (ET) under reduced flow rate, much lower than regular flow rate during air inhalation. This will allow the droplets to avoid inertial deposition and to reach the surface of damaged bronchial tree. As demonstrated by recent computational and experimental works done in our group [Mazela et al., 2011; Penconek et al, 2012], ETs with the internal diameter larger than 4 mm will be penetrated by droplets larger than 20 μm if the aerosol flow through the tube is maintained at the level of 2-3 ml/min. Therefore, it is possible to generate the bio-aerosol to a sterile holding chamber, and then push it slowly through the ET to the lungs of an intubated patient, Figure 11. Although the proposed procedure is invasive, it should be applicable in patients suffering from serious lung damage who are routinely intubated and artificially ventilated.

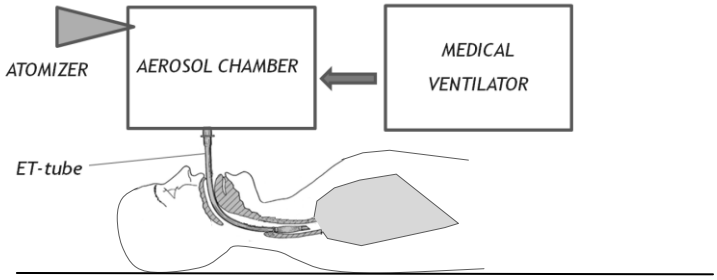

Figure 11. The proposed concept of bronchial delivery of large droplets of sprayed bio-colloid.

It can be concluded that the results of this model research, together with the proposed innovative concepts of aerosol delivery to the bronchial tree, allows to judge the proposed idea of application of sprayed bio-colloid in pulmonary therapy as feasible from the technical perspective. This opens an opportunity for further research on atomization of more specific cellular colloids, e.g. containing stem cells which might be used in lung regeneration (Pojda, 2010) or even in the treatment of other pulmonary diseases (Bonfield and Caplan, 2010).

Acknowledgments

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