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Basic Research

Endoscopic atomization of mesenchymal stromal cells: *in vitro* study for local cell therapy of the lungs

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ABSTRACT

Background aims: Cell-based therapies of pulmonary diseases with mesenchymal stromal cells (MSCs) are increasingly under experimental investigation. In most of these, MSCs are administered intravenously or by direct intratracheal instillation. A parallel approach is to administer the cells into the lung by endoscopic atomization (spraying). In a previous study, the authors developed a flexible endoscopic atomization device that allows administration of respiratory epithelial cells in the lungs with high survival.

Methods: In this study, the authors evaluated the feasibility of spraying MSCs with two different endoscopic atomization devices (air and pressure atomization). Following atomization, cell viability was evaluated with live/dead staining. Subsequent effects on cytotoxicity, trilineage differentiation and expression of MSC-specific markers as well as on MSC metabolic activity and morphology were analyzed for up to 7 days.

Results: MSC viability immediately after spraying and subsequent metabolic activity for 7 days was not influenced by either of the devices. Slightly higher cytotoxicity rates could be observed for pressure-atomized compared with control and air-atomized MSCs over 7 days. Flow cytometry revealed no changes in characteristic MSC cell surface marker expression, and morphology remained unchanged. Standard differentiation into osteocytes, chondrocytes and adipocytes was inducible after atomization.

Conclusions: In the literature, a minimal survival of 50% was previously defined as the cutoff value for successful cell atomization. This is easily met with both of the authors' devices, with more than 90% survival. Thus, there is a potential role for atomization in intrapulmonary MSC-based cell therapies, as it is a feasible and easily utilizable approach based on clinically available equipment.

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Introduction

Administration of mesenchymal stromal cells (MSCs) has been proposed as cell therapy for various lung diseases [1,2]. There are over 200 pre-clinical studies (rodents, large animals, explanted human lungs) demonstrating efficacy in a wide range of lung injury/disease models [2–7]. Comparably, there has been a steady increase in clinical investigations of MSC-based cell therapies in lung diseases

and critical illnesses [7,8]. All of these have demonstrated safety, and a recent industry-sponsored trial has demonstrated efficacy in acute respiratory distress syndrome [9]. In most of the pre-clinical and clinical studies, MSCs were injected intravenously. For this administration route, systemic distribution of cells was shown to have the highest cell retention in lung capillaries [10]. Although an ongoing concern with intravenous MSC administration is emboli in the pulmonary vascular system, particularly in diseased lungs [11], there has been no evidence of this in clinical trials to date [12,13].

In parallel approaches, several groups have investigated direct intratracheal instillation of MSCs and have found similar efficacy compared with intravenous administration in several pre-clinical disease models [5,14,15]. However, a significant disadvantage of

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intratracheal instillation is the delivery of liquid to an already damaged pulmonary system. The authors propose an alternative approach utilizing endoscopic cell atomization (spraying). With this technology, the cell suspension can be administered in a minimal amount of liquid while allowing a better distribution in the lung compared with direct intratracheal instillation [16].

However, the feasibility of cell atomization depends on a number of factors, including hydrostatic, shear and elongation stresses, each of which may result in cell damage [17–21]. Thus, not all atomization devices are feasible for application with cells and have to be tested individually depending on needed cell type and planned application. Veazey *et al.* [22] defined a minimal survival of 50% for evaluation of an atomization device. The authors suggest a much higher survival of at least 90% for cell therapy approaches. The general applicability of endoscopic atomization with a high cell viability has already been proven successfully for respiratory epithelial cells to coat biohybrid stents *in situ* [23]. This process is also suitable for application in humans.

Two commonly utilized atomization approaches are air- and pressure-based systems. In pressure atomizers, the liquid is atomized by passage through a small orifice. Here a pressure drop causes a high velocity such that a spray is produced from the liquid stream. Air atomizers are twin-fluid atomizers in which air with high flow velocities is used to produce a spray from a liquid stream [24]. These approaches were compared for effects on MSC survival, metabolic activity, apoptosis and necrosis, morphology, trilineage differentiation capacity and expression of typical MSC markers.

Methods

Cell culture

Human bone marrow-derived MSCs obtained from normal volunteers were kindly provided by D. Sumstad and Professor D. McKenna, University of Minnesota Medical Center, Minneapolis, MN, USA, through the National Institutes of Health (National Heart, Lung, and Blood Institute Production Assistance for Cellular Therapies program), and by Professor Wolfgang Wagner, Stem Cell Biology and Cellular Engineering, RWTH Aachen University, Aachen, Germany. Use of the cells after patients' informed consent was approved by the local ethics committee of the Medical Faculty of RWTH Aachen University (EK300/13).

Cells were cultured in Mesenpan culture medium (PAN-Biotech) with 2% fetal calf serum (Gibco) and 1% antibiotic/antimycotic (PAN-Biotech) and incubated at 37°C with 5% carbon dioxide (CO₂) in a humidified incubator. Cells up to passage five were used. For atomization experiments, MSCs were washed with phosphate-buffered saline (PBS) (Gibco), detached with trypsin/ethylenediaminetetraacetic acid 0.05/0.02% solution (PAN-Biotech) and resuspended in PBS at the indicated concentrations.

Atomization setup

Two different atomization principles were compared, a pressure and an air atomizer, as described earlier (Figure 1A,B). For air atomization, an air stream of pressurized ambient air was applied via the endoscope's working channel with defined flow and pressure (Table 1). For pressure atomization, droplets were achieved by a nozzle at the catheter's tip, which caused a pressure drop in the liquid during ejection. Here no airflow was applied. For both setups, the cell suspension was dispensed with a syringe pump (Landgraf Laborsysteme) placed on a shaker (Heidolph) to prevent cell sedimentation in the syringe.

Effects on MSC survival

MSCs ($n = 3$ individual donors) were resuspended at a concentration of 2×10^5 cells/mL PBS and stained with Calcein AM $2 \mu\text{mol/L}$ in PBS (AAT Bioquest, Sunnyvale, CA, USA) for at least 20 min at 37°C with 5%

CO₂ in a humidified incubator. Cells were either pipetted (control) or atomized (air and pressure atomization) to a polypropylene beaker with an approximately 6-cm diameter and a distance of 3 cm. Directly after spraying, the cell suspension was transferred with a pipette to 12-well plates. Then, propidium iodide was added ($2 \mu\text{g/mL}$) and cells were imaged with a fluorescent microscope (Axio Observer Z1 or Axio Zoom.V16; Carl Zeiss). In a second experiment, pooled cells from three different donors were resuspended at concentrations of 1×10^5 cells/mL, 2×10^5 cells/mL, 5×10^5 cells/mL, 1×10^6 cells/mL and 4×10^6 cells/mL and atomized as described earlier to evaluate the influence of different cell concentrations on cell survival. For analysis, the cell suspensions were diluted with PBS to achieve the same concentration in all wells. Four technical replicates were analyzed.

Effects on subsequent MSC behavior

For all assays, MSCs were processed and atomized at a concentration of 2×10^5 cells/mL as described earlier. For morphology, expression of characteristic MSC cell surface markers and trilineage differentiation, three individual MSC donors were used and assessed separately ($n = 3$ biological replicates). For metabolic activity, apoptosis and necrosis, cells from three individual donors were processed independently and pooled afterward. These experiments were carried out with five technical replicates.

Morphology

MSCs were seeded in culture flasks and images taken with a phase-contrast microscope (Axiovert 40c and AxioCam ERc 5s; Carl Zeiss) daily for 7 days. Medium was changed three times per week. To assess differences, four cell culture-experienced scientists evaluated the morphology based on two characteristics, eccentricity and compactness, on a scale between 1 and 5. They evaluated blinded phase-contrast images for pipetted controls and air- and pressure-atomized MSCs with $n = 3$ independent donors for day 2, day 4 and day 7. For eccentricity, cells with a circular shape were judged as a 1, whereas elongated and spindle-shaped bodies were judged as a 5. For compactness, the amount of cell extensions was evaluated as a 1 for many extensions and a 5 for cells without extensions.

Metabolic activity

An XTT (2,3-bis[2-methoxy-4-nitro-5-sulphophenyl]-2H-tetrazolium-5-carboxanilide) assay (Roche Diagnostics) was carried out according to the manufacturer's instructions to evaluate whether atomization influenced the metabolic activity of MSCs. On day 1, day 3 and day 7, the XTT reagent/coupling solution mixture (50:1) was added to each well; absorption was measured at 450 nm after 2 h of incubation at 37°C with an Infinite M200 plate reader (Tecan).

Apoptosis

To evaluate whether atomization induced apoptosis, a Caspase-Glo 3/7 assay (Promega) was carried out according to the manufacturer's instructions. On day 1, day 3 and day 7, 100 μL of the test reagent was added to the wells and mixed for 30 seconds and luminescence measured after 30 min.

Necrosis

A ToxiLight cell lysis assay (Lonza) was used according to the manufacturer's instructions to test whether cell atomization resulted in cell necrosis. On day 1, day 3 and day 7, 100 μL of the test reagent was added to the wells and a 1-second integrated reading done after 5-min incubation.

Expression of characteristic MSC cell surface markers

MSCs were analyzed by flow cytometry directly before and 1 week after atomization. MSCs were detached as described earlier and stained with fluorescently labeled antibodies for CD11b, CD34, CD45,

Figure 1. Endoscopic atomization principles and setup. (A) Pressure atomization: catheter (top) and principle (bottom). (B) Air atomization: catheter in bronchoscope (top) and principle (bottom). (C) Experimental setup. (Color version of figure is available online).

CD73, CD79 α , CD90, CD105 and HLA-DR (all BD Biosciences) as defined by Dominici *et al.* [25]. Evaluation of marker expression was carried out with a FACSCanto II (BD Biosciences) and results evaluated with FlowJo v10 software.

Trilineage differentiation

To analyze the differentiation capacity of MSCs after atomization, the cells were differentiated into chondrocytes, adipocytes and osteocytes with respective differentiation media. To analyze whether the processing itself induced differentiation, cells were cultured under the same conditions with Mesenpan culture medium (PAN-Biotech) and stained with the respective dyes. This experiment was carried out with $n = 3$ independent donors for pipetted controls and air- and pressure-atomized MSCs.

For chondrogenesis, cell pellets of $2.5\text{--}5 \times 10^5$ cells were produced by centrifugation at 300 g for 10 min in 15-mL tubes and cultured according to the manufacturer's instructions with MesenCult-AFC chondrogenic differentiation medium (STEMCELL Technologies) for 27 days at 37°C with 5% CO₂. Afterward, pellets were fixed in formaldehyde, dehydrated and embedded in paraffin. Samples were cut into 6- μ m sections and stained with Alcian Blue. Images were taken with a bright-field microscope (Axio Imager.D1; Carl Zeiss).

For adipogenesis, cells were seeded at a density of 1×10^4 cells/cm² in wells of a 12-well plate and cultured according to the manufacturer's instructions with StemPro adipocyte differentiation medium (Thermo Fisher Scientific) for 14 days at 37°C with 5% CO₂. Afterward, cells were fixed with formaldehyde and stained with Oil Red O. Images were taken with a phase-contrast microscope.

For osteogenesis, cells were seeded at a density of 5×10^6 cells/cm² in wells of a 12-well plate and cultured according to the manufacturer's instructions with StemPro osteocyte differentiation medium (Thermo Fisher Scientific) for 16 days at 37°C with 5% CO₂.

Table 1
Parameters for air and pressure atomization.

Parameter	Pressure atomization	Air atomization
Catheter	Olympus PW-6P-1	Olympus PW-2L-1
Volume flow of cell suspension	25 mL/min	25 mL/min
Pressure in syringe during ejection	0.65 bar (9.4 psi)	0.21 bar (3.1 psi)
Volume flow of air	N/A	7 L/min
Air pressure	N/A	1.8 bar (26.1 psi)
Distance to substrate	3 cm	3 cm

N/A, not applicable.

Afterward, cells were fixed with formaldehyde and stained with Alizarin Red. Images were taken with a phase-contrast microscope.

Statistical analyses

Raw data were processed using Excel Professional Plus 2016 (Microsoft Corporation, Redmond, WA, USA). Graphics and statistics were produced using Prism 9.0 (GraphPad Software, San Diego, CA, USA). Values are given as mean $\pm$ standard deviation. Statistical significance was tested in comparison to the pipetted control group. Statistical significance of cell survival was tested using one-way analysis of variance with Dunnett's multiple comparison. For assays and evaluation of flow cytometry and morphology assessment, two-way analysis of variance with Dunnett's multiple comparison was used. $P < 0.05$ was considered statistically significant.

Results

Atomization had no deleterious effects on cell survival

To prove general suitability of the two different spraying setups, cell survival directly after spraying was evaluated using live/dead staining with Calcein AM and propidium iodide. In all experiments, pipetted cells served as control. Cell survival was 91.5% and 95.0% of the control group for air- and pressure-atomized cells, respectively (Figure 2A). The slight decrease in survival for both experimental groups was not statistically significant.

Ideally, cells should be applied in high concentrations to limit the amount of liquid introduced to the lungs. Thus, cell suspensions with concentrations from 1×10^5 cells/mL to 4×10^6 cells/mL were air- and pressure-atomized. Here no change in survival was observed for any of the tested concentrations (Figure 2B,C). Thus, cell concentration during atomization did not influence cell survival.

Atomization did not alter MSC morphology over 1 week of subsequent culture

MSC morphology was assessed for 7 days after cell atomization to evaluate whether changes or signs of senescence occurred (Figure 3). In all groups, MSCs remained spindle-shaped. Changes in granulation were not visible. No differences between the pipetted control group and atomized cells could be seen. Statistical assessment of the blinded evaluation by experienced reviewers revealed an increase in cell eccentricity

Figure 2. Cell survival after cell atomization. Cell survival was evaluated with Calcein AM and propidium iodide directly after atomization. (A) Cell survival was >91% of the pipetted control group for all experimental groups with $n = 3$ individual donors. For cell concentrations between 1×10^5 and 4×10^6 , absolute cell survival rates were >89% after air atomization (B) and >88% after pressure atomization (C), with pooled cells from three individual donors measured as four technical replicates. Bars show mean values, dots indicate the individual values for the replicates and error bars represent the standard deviation. $P < 0.05$ was considered statistically significant. (Color version of figure is available online).

and a slightly decreasing cell compactness with time, but no significant differences between the experimental groups were observed (see supplementary Figure 1). In addition, it was observed qualitatively that all groups showed similar proliferation over the course of 1 week.

Atomization had no effect on MSC metabolic activity, but slightly increased apoptosis rates were seen

Cytocompatibility of the two different atomization processes was evaluated by assessing metabolic activity, apoptosis and necrosis for up to 7 days after spraying (Figure 4). Metabolic activity similarly increased from day 1 to day 3 in all groups and remained stable afterward. The cell seeding density for the assays differed from the density used for the morphology studies shown in Figure 3. Slight but statistically significant increases in apoptosis were observed for pressure-atomized MSCs compared with the pipetted control group but not for air atomization on day 1, day 3 and day 7. In general, necrosis levels comparably increased for all groups from day 1 to day 3, with lower values observed on day 7. However, there was a significant increase in necrosis on day 3 for pressure-atomized MSCs compared with the pipetted controls.

Atomization did not alter cell surface expression of characteristic MSC markers

Expression of characteristic MSC surface markers was analyzed before and 1 week after atomization with flow cytometry (Figure 5).

In all cases, CD73, CD90 and CD105 were expressed in more than 95% of cells, and CD11b, CD34, CD45, CD79 α and HLA-DR were expressed in <2% of cells, without any differences between pipetted controls (both on day 0 and day 7) and atomized cells. The values for all samples can be seen in supplementary Table 1.

Trilineage differentiation was successful after atomization

Differentiation of MSCs into adipocytes, osteocytes and chondrocytes was evaluated by culturing the pipetted control group and air- and pressure-atomized cells in the respective differentiation media. In Figure 6A, adipocytes are shown by Oil Red O staining of lipoproteins in lipid vesicles of the cells for all experimental groups cultured with adipogenesis medium. Although differentiation occurred mostly in areas with a high cell density, it was generally slightly qualitatively lower in pressure-atomized cells. MSCs, in which differentiation was not stimulated by adipogenesis medium, did not show lipoprotein-positive staining. In Figure 6B, osteocytes are shown by Alizarin Red staining of calcium deposits in the cells for all experimental groups cultured with osteogenesis medium. There was no obvious qualitative difference observed between the groups. MSCs cultured with proliferation medium did not show staining for calcium deposits. Figure 6C shows the differentiation of MSC pellets into cartilage by Alcian Blue staining. For all groups, induced samples showed an intense blue staining. Thus, glycosaminoglycans were abundant in the pellets, consistent with differentiation into cartilage.

Figure 3. MSC morphology after atomization. MSCs of control group (pipetted cells) and experimental group are spindle-shaped, without signs of senescence or cell death.

Figure 4. Metabolic activity, apoptosis and necrosis after atomization. (A) Metabolic activity was measured by tetrazolium salt cleavage, and no differences between the groups were seen. (B) Apoptosis was analyzed by caspase-3 and -7 activity and showed a significant increase for pressure atomization that was still in the same order of magnitude as the other groups. (C) Necrosis was evaluated by adenylate kinase release and showed a significant increase only on day 3 for pressure atomization. Bars show mean values, dots indicate the individual values and error bars represent the standard deviation for pooled cells from three individual donors measured as five technical replicates. $P < 0.05$ was considered statistically significant. (Color version of figure is available online).

Non-induced samples did not show Alcian Blue-positive staining and had a higher cell density than differentiated samples. There was no obvious qualitative difference observed between the experimental groups.

For the trilineage differentiation, although all lineages were successful, the extent of differentiation varied for the different donors. Thus, in the figures, representative images of one donor are shown to allow comparison between the different experimental groups.

Discussion

In this study, the authors assessed the feasibility of MSC atomization for use in pulmonary cell therapy, as there is a need to overcome the low efficacy of cell therapy in clinical trials to date [11,26]. The aerosolization approach might be more feasible than direct instillation [16], in particular, as cell application as a spray allows the

reduction of liquid instilled to the lung, which could further damage diseased lung tissue. Notably, the authors found that atomizing MSCs in suspensions of up to 4×10^6 cells/mL had no deleterious effect on cell survival, metabolic activity or differentiation potential and thus could potentially provide therapeutic doses to the lungs. The authors also speculate that the concentration can be further increased without negative influence on the cells.

To optimize aerosol-based MSC delivery, two different atomization types were compared: pressure and air atomization. As the name suggests, in pressure atomizers, the kinetic energy for aerosol production is provided by pressure energy or, rather, pressure drops. The smaller the orifice through which the liquid has to pass, the finer the atomization [24]. With the nozzle used here, high survival is reached while maintaining a fine aerosol with a flow velocity of 25 mL/min. Apparently, neither the high pressure in the nozzle nor the pressure drop when exiting the nozzle caused any significant

Figure 5. Flow cytometry results before and 1 week after cell atomization. (A) CD73, CD90 and CD105, which should be expressed in $\geq 95\%$ of MSCs. (B) CD11b, CD34, CD45, CD79 α and HLA-DR, which should be positive in $\leq 2\%$ of MSCs, as defined by Dominici et al. [25]. Note the different y-axis scales. Dashed lines indicate respective cutoff values. Bars show mean values, dots indicate the individual values for the three individual donors and error bars represent the standard deviation. $P < 0.05$ was considered statistically significant. (Color version of figure is available online).

Figure 6. Differentiation of MSCs into adipocytes, osteocytes and chondrocytes. (A) Oil Red O: positive staining was observed in all experimental groups cultured with adipogenesis medium, whereas no staining was observed for cells cultured with proliferation medium. (B) Alizarin Red: positive staining was observed in all experimental groups cultured with osteogenesis medium, whereas no staining was observed for cells cultured with proliferation medium. (C) Alcian Blue: positive staining was observed in all experimental groups cultured with chondrogenesis medium, whereas no staining was observed for pellets cultured with proliferation medium. (Color version of figure is available online).

damage with regard to cell survival. Of the different parameters assessed, no effects on viability, morphology, metabolic activity, possibility of trilineage differentiation or expression of characteristic cell surface markers were observed compared with non-atomized cells. However, there was an increase in apoptosis on all days and necrosis at day 3, and the extent of adipogenesis was reduced for pressure-atomized cells.

Air atomizers are two-component atomizers in which an air stream provides the energy for atomization. With these types of atomizers, finer sprays can be produced than that seen with pressure atomizers [24]. The high amount of administered air as well as its velocity has to be taken into account, especially when considering the system for therapy of the lungs. For this approach, high survival and no changes in viability, morphology, metabolic activity, apoptosis, necrosis, trilineage differentiation or expression of cell surface markers compared with non-atomized cells were observed.

Shear and elongation stresses are felt to be responsible for cell damage during atomization [27]. Both stresses occur mostly because of liquid films draining during droplet formation. Thin liquid layers form during atomization just before the liquid stream breaks into droplets. These films then drain because of external forces resulting from surface

tension or flow. Draining liquid films have been noted to cause cell damage in bubble-column bioreactors used for suspended cell culture [28]. Next to shear and elongation stresses, cell impact onto the substrate can influence cell behavior. In this study, cells were atomized with a low distance of 3 cm to the substrate (polypropylene). In other studies, higher distances from the aerosolization device to the substrate resulted in lower cell survival [29,30]. Substrate stiffness was previously shown to not influence cell survival [31]. In addition, there is a high level of hydrostatic pressure acting on the cells before atomization, especially with the pressure atomization process. Even though the applied pressure is more than twice as high as physiological pressures, it seems to be well tolerated by the cells. This can be explained by the following reasons: First, the pressure is applied for only a minimal amount of time; Second, hydrostatic pressures are assumed to have a minimal effect on cells, as they are incompressible [32].

Regarding MSC morphology, no changes were observed between control and atomized cells. No signs of cell death or senescence were seen. Senescent cells stop proliferating, and all somatic cells reach this state at some point in *in vitro* culture; for MSCs, this is a critical factor in their differentiation capability and immunomodulatory capacity [33,34]. MSC characteristics after atomization were further

assessed by flow cytometry, and no change in expression of characteristic MSC surface markers, as defined by Dominici *et al.* [25], was seen. In addition, trilineage differentiation into adipocytes, osteocytes and chondrocytes with stimulation by the respective differentiation media was successful for all experimental groups. By contrast, without differentiation media, MSCs did not differentiate as a response to the atomization process. This is of critical importance, as mechanical stresses have previously been shown to induce embryonic stem cell differentiation [35]. This effect was not observed here, as the MSCs were exposed to the stresses for very short durations during atomization only. As all experiments were carried out with three independent donors, differences in behavior were observed between the cells from different donors, especially with regard to trilineage differentiation. Inter-donor variability is an important topic in studies using MSCs [36], such that standardization and use of a sufficient number of independent donors need to be considered. Additionally, evaluation of pooled cells should be avoided in the future so that each cell line's reaction can be analyzed separately.

Other studies have described MSC atomization with hand-held atomization devices for treatment of wounds, gastric perforation and airway inflammation in asthma [37–39]. In a study by Tritz-Schiavi *et al.* [40], 3 days after MSC atomization utilizing an airbrush system, initial survival of approximately 50% was observed. However, survival increased from day 7 on. In the researchers' airbrush system, the cell suspension was fed from a reservoir mounted below the air stream. High air velocities are needed to produce negative pressure, which draws the solution off the reservoir. These pressures are probably responsible for the comparably low survival. An advantage of this system compared with gravity-fed airbrush systems is a bigger reservoir.

Veazey *et al.* [22] defined a minimal survival of 50% for cell atomization devices to achieve sufficient cell numbers in treatment of acute or chronic skin loss. This requirement of 50% survival is easily met with both of the authors' systems directly after atomization. Nevertheless, the authors suggest higher survival of at least 90% for pulmonary cell therapy approaches. In the authors' study, we observed survival of almost 90% and thus suggest that both systems are suitable for cell therapy with regard to cell survival.

A study by Halim *et al.* [37] showed that aerosolized MSCs significantly reduced airway inflammation in an asthma model in rabbits. The researchers used an atomization device, which is suitable for intratracheal administration in small animals, from Penn-Century. However, despite morphological assessment, no evaluation of cell behavior after atomization was shown.

In a study by Kim *et al.* [31], two atomization devices were tested with amniotic membrane-derived MSCs. A microsyringe from Penn-Century similar to the one used by Halim *et al.* [37] failed the *in vitro* tests, as it resulted in droplet sizes smaller than cell diameters, and cells appeared shredded after processing. In addition, using an atomizer from Teleflex, which is utilized for drug administration in human upper airways, survival of about 80% was reported, which was comparable to the control [31]. For both of the devices used in the authors' current study, cell survival was higher than 90%. As Kim *et al.* did not observe a significant decrease in survival, with approximately 85% surviving cells in the controls, a higher total survival could be achieved when using cell suspensions with a higher viability to begin with. Nevertheless, cell density was lower for atomized cells, and hence the duration until confluence was longer, which was not seen in the authors' current study. Kim *et al.* reported that no changes in apoptosis were observed on day 3. For the pressure atomizer used in the authors' current study, slightly increased apoptosis rates were observed on all days. For the authors' air atomizer, no changes in metabolic activity, apoptosis or necrosis were observed. Thus, cell application efficiency by means of cell density after atomization seems to be better for both of the authors' devices, whereas the Teleflex atomizer causes less cell damage compared with the authors' pressure atomizer.

The devices from Penn-Century and Teleflex as well as the authors' pressure atomizer produce fine sprays without pressurized air, which might be advantageous compared with twin-fluid atomizers. Air atomizers can induce lung damage as a result of administration of high amounts of air with high flow velocities. However, cell damage was slightly higher for the cells processed with the pressure atomizer.

Most droplets $\geq 2 \mu\text{m}$ collect in the upper airways when inhaled or at the first bifurcations when administered in the trachea [41,42]. This is conceivably a problem for atomizers like the ones presented in the studies by Kim *et al.* [31] and Halim *et al.* [37]. In the authors' current study, we present two options for bronchoscopic spray administration in humans, which can be used to aerosolize cells locally down to human segmental bronchi. The catheters have a length of $\geq 150 \text{ cm}$. Thus, specific lung areas can be targeted directly to increase delivery efficiency. At the same time, atomization may allow better distribution of the cell suspension throughout the lungs.

For all devices, attention has to be given to ejection of cell suspensions, as velocity influences spray patterns and droplet sizes and can therefore alter cell survival. As suggested by Kim *et al.* [31], syringe pumps should be used in all future applications to guarantee reproducible results.

One of the limitations of this study is the lack of *in vivo* data. Here the authors focused on *in vitro* studies and intensively analyzed MSC behavior after atomization as an important basis for future studies and proved general suitability of the concept with our setup. Ideally, future *in vivo* studies should be accomplished in large animal models to provide a geometry similar to human lungs, as this is particularly important for judging cell distribution.

Conclusions

In this study, an endoscopic air atomizer and an endoscopic pressure atomizer were proven suitable for MSC administration, with potential applications in intrapulmonary cell therapy. High viability and successful trilineage differentiation were observed. In future studies, cell distribution in the lungs and effectiveness as well as efficiency will be assessed in pre-clinical models.

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Declaration of Competing Interest

The authors have no commercial, proprietary or financial interest in the products or companies described in this article.

Author Contributions

Conception and design of the study: ALT and SJ. Acquisition of data: ALT, FEU and MH. Analysis and interpretation of data: ALT, CGC and DJW. Drafting or revising the manuscript: ALT, FEU, MH, CGC, SJ and DJW. All authors have approved the final article.

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Supplementary materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.jcyt.2020.12.010.

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