Efficiency assessment of irrigation as an alternative method for improving the regenerative potential of non-healing wounds

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Abstract
The application of mesenchymal stem/stromal cells (MSC) in regenerative medicine offers hope for the effective treatment of incurable or difficult-to-heal diseases. However, it requires the development of unified protocols for both safe and efficient cell acquisition and clinical usage. The therapeutic effect of fat grafts (containing stem cells) in non-healing wounds has been discussed in previous studies, although the application requires local or general anaesthesia. The treatment of MSC derived from adipose tissue (ASC) could be a less invasive method, and efficient delivery could lead to more favourable outcomes, which should encourage clinicians to use such therapeutic approaches more frequently. Therefore, the aim of this study was to optimise the methods of ASC isolation, culture and administration while maintaining their high survival, proliferation and colonisation potential. The ASC were isolated by an enzymatic method and were characterised according to International Society for Cellular Therapy and International Federation for Adipose Therapeutics and Science guidelines. To assess the opportunity to obtain a sufficient number of cells for transplantation, long-term cell cultures in two oxygen concentrations (5% vs. 21%) were conducted. For these cultures, the population doubling time, the cumulative time for cell population doublings and the rate of cell senescence were estimated. In a developed and pre-defined protocol, ASC can be efficiently cultured at physiological oxygen concentrations (5%), which leads to faster proliferation and slower cell senescence. Subsequently, to select the optimal and minimally invasive methods of ASC transplantation, direct cell application with an irrigator or with skin dressings was analysed. Our results confirmed that both the presented methods of cell application allow for the safe delivery of isolated ASC into wounds without losing their vitality. Cells propagated in the described conditions and applied in non-invasive cell application (with an irrigation system and dressings) to treat chronic wounds can be a potential alternative or supplement to more invasive clinical approaches.

KEYWORDS
cell therapy, irrigation, mesenchymal stem/stromal cells, non-healing wounds
1 | INTRODUCTION

The problem of non-healing wounds affects 200 million people around the world. These patients are mainly elderly, suffer from diabetes, advanced venous insufficiency and experienced radiation therapy or paraparesis. Chronic wounds, in contrast to acute wounds (caused by trauma or surgical interventions), do not heal according to the typical pattern, developing inflammation, granulation and remodelling associated with the formation of collagen tissue. These wounds develop a state of chronic inflammation, which minimises the regenerative capacity of the tissue. Chronic wound healing is impaired by local factors, such as ischemia and/or infection, and systemic factors, such as advanced age, malnutrition, diabetes or kidney disease. Good clinical practice should eliminate or reduce the impact of these factors; however, it is often impossible. In addition to local and general factors, the chronic wound environment is characterised by a reduction in the number of proliferative stimulants, an imbalance between proteolytic enzymes and their inhibitors, accelerated cell senescence and increased production of inflammatory cytokines. In the treatment of non-healing wounds, various surgical techniques, including extensive surgical or enzymatic debridement, innovatory dressings, negative pressure therapy, skin grafts or reconstructive methods with flap transfers, have been proposed. Unfortunately, the treatment is prolonged, expensive and not always efficient. The application of mesenchymal stem/stromal cells (MSC) in regenerative medicine offers hope for a combination of standard treatment with additional therapies. MSC are thought to evoke a therapeutic effect through two mechanisms of action: restoration and adjuvant healing.

In response to environmental signals, MSC are not only able to differentiate into the cells of the tissue where they reside but can also differentiate into cells developed from other germ layers.

Important mechanisms of MSC action are associated with their strong adjuvant properties. Due to their immunomodulatory potential, MSC have the ability to inhibit the progression of inflammatory processes. Therefore, the selection of cells for regenerative therapy requires appropriate adaptation of the cell isolation source to the intended therapeutic effect.

Adipose tissue is one of the most popular sources of MSC and is promising for chronic wound treatment. Comparing mesenchymal stem cells isolated from adipose tissue (ASC, adipose stem/stromal cells) to bone marrow-derived somatic stem cells (BMSC), which were first used for therapeutic purposes, ASC constitutes 2% of the vascular support fraction, while BMSC constitutes only 0.002% of the heterogeneous bone marrow-derived population. Both stem cell populations, BMSC and ASC, are of mesenchymal origin, but their adjuvant properties differ.

The therapeutic effect of stem cells has been confirmed in numerous independent studies, but for the therapy, a sufficiently large number of cells is essential. Maintaining MSC in culture in vitro for their propagation leads to increased cell senescence and a decrease in their adjuvant properties, thereby causing the loss of their therapeutic potential.

The aim of our study was to establish a protocol for ASC isolation from human subcutaneous adipose tissue in clinical settings, to optimise the culture conditions for increasing their propagation in vitro while maintaining their initial potential for proliferation and slowing their senescence in culture, and to establish a non-invasive method for their application in the clinic.

The ability to control the proliferation and differentiation of cell culture is connected to external factors, that is, the composition of the culture medium, pH or oxygen concentration. Considering our previous experiments and reports from the literature, we are convinced that one of the crucial factors that can protect cells in culture from unfavourable processes is the appropriate concentration of oxygen. It has been assumed that the optimal oxygen concentration in culture should be 21%, which is the same as the atmospheric concentration. However, intratissue oxygen concentrations differ from 2% to 9% O2 (14–65 mm Hg). This range has been recognised as physiological normoxia, called physioxia. It is postulated that for both bone marrow and adipose-derived MSC, the physiological oxygen concentration is a key factor in maintaining the undifferentiated character of cells.

ASC transplantation to non-healing wounds needs to be non-invasive and affordable. Therefore, we used commercial, easily accessible and relatively inexpensive surgical dressings, which were populated with ASC. This method of administration into small but penetrating wounds allows longer exposure to the growth factors and immunomodulators secreted by the cells and, at the same time, protects both the transplanted cells and the wound from adverse environmental conditions, that is, dry air or mechanical trauma. Different methods must be used for extensive and superficial wounds, which are stimulated to fill with granulated tissue before skin graft application. In this case, the cells need to be seeded into a broad area, often several times, and preferably non-invasively. For this purpose, we propose the usage of accessible and commonly used dental irrigators.

A general overview of the study steps is presented in Figure 1.

2 | MATERIALS AND METHODS

Adipose tissue and skin biopsy samples were obtained from the Department of Plastic Surgery, Medical Centre for Postgraduate Education in Warsaw. All the described procedures were approved by the local ethics committee.

2.1 | Isolation of adipose-derived mesenchymal stem/stromal cells

Fat tissue was harvested from the abdomen or inner thighs after infiltration with Klein solution (based on lactated Ringers solution with lidocaine and epinephrine) under local anaesthesia. To preserve the regenerative properties of cells, the fat collection was performed at low pressure (250 kPa) using syringe liposuction. The liposapirate was immediately transported to the Cell Culture Laboratory for further isolation procedures. The ASC were isolated using an enzymatic method with collagenase VI (Serva, collagenase NB6 Grade, cat.
no. 17458). Equal amounts (50 ml) of lipoaspirate collagenase VI (0.4 PZ U/ml) were added (the final enzyme concentration was 0.2 PZ U/ml) and incubated at 37°C on an orbital shaker with a rocking speed of 60 cycles/min for ~45 min. In the next step, the stromal vascular fraction (SVF) was centrifuged for 20 min at 200 g to separate the cells. The supernatant was removed, and the cells were suspended in MSCGM (Lonza, cat. no. PT-3238), seeded in six-well plates and cultured at 37°C and 5% CO₂ in two oxygen concentrations: 21% and 5%. The growth medium was changed every 2–3 days.

2.2 | Isolation of fibroblasts

The skin biopsy samples were washed with PBS supplemented with 1% antibiotics (an antibiotic and antifungal, Gibco, cat. no. 15240), placed on a Petri dish and cut with a sterile scalpel into 1 mm × 1 mm fragments. These fragments were treated with a 0.075% solution of collagenase 1A (Serva), incubated at 37°C for 3 h on an orbital shaker for ~60 cycles/1 min, and then transferred into a 24-well plate. The cell culture was maintained in high-glucose Dulbecco’s medium (Gibco) supplemented with 20% foetal bovine serum (Biosera) and 1% penicillin/streptomycin solution (Gibco) at 37°C, 21% O₂ and 5% CO₂. The medium was replaced every 2–3 days. The FBS concentration was decreased to 10%. After 2 weeks, tissue fragments were removed from the plate, and fibroblasts that migrated from biopsy samples grew on a plastic surface in a monolayer and were trypsinized and collected.

2.3 | Immunocytochemistry analysis

For immunocytochemical analysis, cells were seeded on 24-well plates covered with poly-lysine (Sigma-Aldrich) at a density of 5 × 10⁵ cells/cm². When the culture was subconfluent, the growth medium was removed, the cells were rinsed with PBS, and then, the cultures were fixed with 4% PFA for 15 min and washed again with PBS. To label intracellular marker proteins, cells were treated with 0.1% Triton X-100 solution for 15 min, and non-specific bindings were blocked with 10% goat serum (Sigma-Aldrich) and 1% bovine serum albumin (Sigma-Aldrich) solution for 1 h at room temperature (RT). Subsequently, primary antibodies against CD73 (1:100), CD90 (1:100), fibronectin (1:500) and vimentin (1:200) were applied overnight at 4°C. The following day, the cultures were washed thoroughly with PBS, and secondary antibodies (Alexa Fluor 488 for green staining or Alexa Fluor 546 for red staining; dilution 1:1000, Invitrogen) were added for 1 h. The cells were rinsed with PBS, and the cell nuclei were stained with Hoechst 33258 dye (2 μg/ml, Sigma-Aldrich) for 15 min. The slides were closed with fluorescence mounting medium (DakoCytomation, S3023) and analysed using an Axio Vert A1 microscope with an AxioCam MRC5 camera (Zeiss) and ZEN software.
2.4 | Flow cytometry analysis

Phenotypic characterisation of the cells was performed using a commercial kit for typical mesenchymal marker identification with a BD StemFlo™ hMSC analysis kit (BD Biosciences). To detach ASC from culture dishes, surface Accutase (BD Biosciences) solution was added, and then the ASCs at a minimum density of $1 \times 10^6$ cells/ml were suspended in a cold buffer designed for flow cytometry (amplifying the fluorescence signal) with BD Phormingen. The cells were labelled for 30 min at RT with fluorochrome-conjugated antibodies directed against the CD90-FITC positive markers CD73-APC and CD105-PerCP-Cy5.5 and the negative biomarkers CD34-PE, CD11b-PE, CD19-PE, CD45-PE and HLA-DR-PE. To exclude non-specific readings and interference, isotype controls and compensation for each fluorochrome were performed. The measurement was carried out with a FACSCalibur II by Becton Dickinson and FACSDivia software. A total of $1 \times 10^5$ events for each sample were analysed. The results are presented as a percentage of positive cells for individual markers relative to isotype controls in the form of a histogram.

2.5 | Mesodermal lineage differentiation

The differentiation potential was determined using a StemPro adipogenesis/chondrogenesis/osteogenesis differentiation kit (Gibco). To evaluate the capability of mesodermal lineage differentiation, ASC were seeded in 24-well plates at a suitable density (for the adipogenesis experiments: $1 \times 10^4$ cells/cm²; for the osteogenesis experiments: $5 \times 10^3$ cells/cm²; and for the chondrogenesis experiments: $1.6 \times 10^3$ cells/cm²) and cultured to subconfluency in MSC growth medium (Lonza) under 21% O₂.

Cartilage differentiation was induced by a chondrogenesis differentiation medium (Gibco). The micromass pellet of cells was generated in the centre of the well plate. After 1 h of incubation at 37°C, the differentiation medium was added, and the culture was continued for an additional 14 days with the medium replaced every 2-3 days. ASC were cultured in differentiation conditions for 14 days (for adipogenesis and chondrogenesis experiments) or 21 days (for osteogenesis experiments). After this time, the cells were fixed with 4% PFA and stained with 10% oil red O solution for 5 min to confirm the differentiation of the cells into adipocytes, with 2% Alizarin red S solution for 2 min to confirm the differentiation of the cells into osteocytes, and with 1% Alcian blue for 15 min to confirm the differentiation into chondrocytes. The preparations were analysed using an Axio Vert 40C microscope (Zeiss) with a Canon G10 camera (Zeiss).

2.6 | Vasculogenic potential of adipose stem or stromal cells

The ability of ASCs to form capillary-like structures was defined based on a tube formation assay performed with a μ-Plate Angiogenesis kit (BD). To perform the test, 10 μl of Matrigel™ (Becton Dickinson) was added to the μ-Plate wells and incubated at 37°C for 30 min. ASCs were labelled with a green CMFDA tracer (Chloromethylfluorescein diacetate, Life Technologies) for 30 min, washed with PBS solution, detached with Accutase solution (Becton Dickinson, C-41310), suspended in standard medium (MSCGM, Lonza) and centrifuged at 1000g for 3 min. Subsequently, the cells were seeded on Matrigel at a density of $5 \times 10^4$ cells/cm² in standard MSCGM and incubated under standard conditions. The capacity of ASC to create tubule-like structures was visualised after 2 and 4 h of incubation with an Axio Vert A1, AxioCam MRC5 camera (Zeiss) and ZEN software.

2.7 | Cell proliferation analysis

2.7.1 | Long-term cell proliferation analysis

In each passage (from the 1st to the 20th passage), the subconfluent ASC were trypsinized, counted and reseeded on a plate at a density of 3000 cells/cm². The parameters characterising ASC proliferation capacity, such as the growth curve, cumulative population doubling (CPD) and population doubling time (PDT), were calculated as described previously. Briefly, proliferation parameters were calculated based on the total cell number at each passage using the following formulas: PDT = $t_0$ × log 2/(log $N_0$ – log $N$) and CPD = $PD_{t_1} + PD_{t_2} + PD_{t_3} + ... + PD_{t_n}$, where $PD_t = \log(N_0/N)/\log 2$. The duration of passage, counted in days, is equal to $t_0$. $N$ is the number of cells obtained at the end of a particular passage, whereas $N_0$ is the number of seeded cells.

2.7.2 | One-week cell proliferation analysis

The growth of living cells during one passage was estimated by the analysis of daily changes in sample metabolic activity with the WST-1 reagent (Roche). ASCs after the 4th passage were seeded on 96-well plates, and for the analysis, the enzyme activity was estimated daily. WST-1 solution (10 μl) was added to each sample and incubated in the dark in an incubator at 37°C for 1 h. After fixation, the cells were washed twice with 1% diacetate, Life Technologies) for 30 min. The fluorescence signal was amplified using a BD Luminex™ detection system, and the fluorescence was measured spectrophotometrically at 420 nm using an Omega plate reader (BMG LABTECH). The curve of cell growth was plotted with time on the x-axis and cell number on the y-axis.

2.8 | Cell senescence assay

Cells were analysed at the 4th, 12th and 22nd passages using a senescence cell histochemical staining kit (Sigma-Aldrich). Cultured cells were washed twice with 1× PBS and fixed with fixation buffer for 6-7 min at RT. After fixation, the cells were washed three times with 1× PBS, and the staining solution was prepared according to the manufacturer’s protocol. The cells were incubated overnight at 37°C. The total
number and blue-stained cells were counted, and the percentage of stem-associated β-galactosidase (SA-βgal)-positive cells was calculated.

2.9 | Coculture of adipose stem or stromal cells with fibroblasts

Fibroblasts were cultured in 24-well plates at a density of $3 \times 10^3$ cells/cm$^2$ under standard conditions: 37°C, 5% CO$_2$, 21% O$_2$, high-glucose Dulbecco’s medium (Gibco) supplemented with 10% foetal bovine serum (Biosera) and 1% penicillin and streptomycin solution (Gibco) until semiconfluence was obtained. Then, the ASC labelled with CMFDA were seeded on the fibroblast layer at a density of $10^5$ cells/cm$^2$. ASC with fibroblasts were cocultured in MSCGM for 48 h.

2.10 | Scratch assay

To evaluate the influence of AS on fibroblast proliferation, a scratch assay was performed on the ASC/fibroblast coculture monolayer. The confluent coculture monolayer was scored with a sterile pipette tip to leave a scratch ~0.4–0.5 mm in width. The experiment was carried out under standard culture conditions 48 h after the scratches were made. To analyse the cells’ ability to migrate into the scratched area, photographs were taken at 0, 24 and 48 h by using an Axio Vert A1 microscope with an AxioCam MRC5 camera (Zeiss). The migrated cells were counted with Zen software (Zeiss).

2.11 | Optimization of adipose stem or stromal cells clinical application with irrigator or dressing usage

To optimise ASC application (prolong the survival and localise precisely) on non-healing skin wounds, three types of wound dressings commonly used in clinical practice, namely, hydrocolloid, gelatin and paraffin dressings, were tested. The dressing fragments (1 cm × 1 cm) were plated into six-well plates and immersed in MSCGM. ASC labelled with CMFDA were seeded on the dressing surfaces at a density of $2 \times 10^5$ cells per fragment. After 24 h of cell culture, cell survival was assessed with propidium iodide (IP, Sigma Aldrich) at a 1 mg/mL concentration.

For rapid, easy and direct ASC application on extensive injury surfaces, a dental irrigator was tested. To determine the optimal parameters, such as dispersion and flow, that ensure optimal cell survival, different parameters were tested. To conduct these experiments, ASC were suspended in 20 ml of MSCGM and added to the fluid container. Next, using a different flow rate and different stream shapes, cells were applied to the plastic plate, and their survival was estimated with trypan blue staining. The results are presented as the percentage of the live cell subpopulation compared to the total cell population. In the next step, the appropriate medium for cell suspension was tested. The ASC were suspended in Ringer’s solution or 0.9% NaCl. Cell survival was estimated as described above.

2.12 | Surgical treatment and stromal vascular fraction application in pilot cases

Chronic wounds were surgically prepared. After washing, mechanical debridement of devitalized superficial layers of wound surfaces was performed with a small surgical spoon, and the outer contaminated layers of wounds were removed. For extended wounds with bone exposition, we performed cortical layer removal and skin graft. Penetrating wounds were cleaned and refreshed only. Autologous fat (200 ml) was harvested from the lower abdomen, and the SVF was isolated as described earlier. External applications of SVF solution (prepared immediately during surgery) were applied with an irrigator (in extended wounds) and in an external dressing (hydrocolloid foam) to cover deep penetrating wounds. In both cases, all obtained cells were applied (~ $9.6 \times 10^5$ cells/ml).

In accordance with in vitro experiments for direct SVF application with an irrigator, the cell fraction after the isolation procedure was suspended in 20 ml of Ringer’s solution (the smallest amount of buffer for irrigator usage). With a diffuse stream and an intermediate third mode of flow, the suspension was dispersed and applied once on the wound surface.

To support deep penetrating wound regeneration, a hydrocolloid dressing was used. The obtained cell fraction was suspended in 5 ml of Ringer’s solution in a syringe and steadily deposited on the dressing surface. Such prepared dressing was located on the wound. The SVF solution was applied twice with a 3-month interval.

2.13 | Statistical analysis

Statistical analysis of the raw data was conducted using GraphPad Prism 5 software. The mean ± SD was calculated for all samples, $n = 4–6$, and significance was determined using Student’s t-test. The notations *$p < 0.05$, **$p < 0.01$ and *** $p < 0.001$ were adopted to report the statistical significance.

3 | RESULTS

3.1 | Adipose stem or stromal cells enzymatic isolation with collagenase VI

The experiments confirmed that the enzymatic method of ASC isolation with collagenase VI for clinical use, slightly modified by our group, is reproducible and efficient. The cells according to the ISCT (International Society for Cellular Therapy) and IFATS (International Federation for Adipose Therapeutics and Science) guidelines, presented a typical fibroblast morphology and MSC surface antigen profile, as shown in Figure 2B. Flow cytometry analysis also confirmed...
the presence of CD-90 (98.6% of cells), CD105 (99.9%) and CD-73 (99.8%) on the ASC surface and a lack of haematopoietic markers, namely, CD34, CD11B, CD19, CD45 and HLA-DR (only in 0.16% of analysed cells; Figure 2A). The ability of the ASC to differentiate toward adipocyte, osteocyte and chondrocyte lineages in vitro was also confirmed (Figure 2C).

**FIGURE 2** Characterisation of the ASC. (A) Flow cytometry analysis showed typical and relatively high expression of specific mesenchymal markers (CD73, CD90 and CD105). Similarly, less than 1% of the ASC expressed negative markers (CD34, CD11b, CD19, CD45 or HLA-DR). (B) Immunocytochemical analysis of the ASC. Scale bar: 50 μM. (C) Multilineage mesodermal differentiation potential of the ASC. A positive differentiation result was noted for the ASC differentiated into bone, adipose and cartilage lineages after oil red O (adipogenesis), Alcian blue (chondrogenesis) and Alizarin red S (osteogenesis) staining, respectively. (D) ASC vasculogenic potential analysis. ASC labelled with CMFDA formed a network, and pictures were taken at two time points: 2 h (left image) and 4 h (right image) after the ASC were seeded on Matrigel. Scale bar: 100 μM.
3.2 | Formation of a vascular network structure

The ability of cells to form capillaries was examined with a Matrigel angiogenesis assay. ASC that had not differentiated into endothelial cells were seeded on ECMatrix gel in a standard MSCGM medium. Spontaneous capillary-like structure formation was observed 2 h after cell injection into ECMatrix gel (an in vitro model of wound stroma). The formation of the mature vascular

![Diagram](image-url)

**FIGURE 3** Proliferation and senescence analysis of ASC cultured under 5% and 21% O₂ conditions. (A) Morphological and SA-β-gal activity changes in ASC during long-term culture. Photos magnified 100×. Scale bar: 200 μm. (B) Growth curve of the ASC. ASC cultured with 5% O₂ proliferated faster. The results showed significantly higher cell numbers per cm² for ASC in the 1st passage and a stable growth phase from the 8th to the 15th passages. (C) ASC population doubling time analysis. The population doubling time was shorter at all passages for the cells cultured with 5% oxygen and significantly shorter at the 1st, 2nd, 4th, 6th—13th, 15th, 17th, and 18th passages. (D) Cumulative population doubling. The CPD was relatively higher for cells cultured with 5% oxygen in the 6th passage and later. (E) One-week ASC growth analysis. ASC presented a three-phase growth rate, which was significantly higher for the cells cultured with 5% oxygen. The results are presented as the mean ± SD of 4–6 experiments; *p < 0.05, **p < 0.01; and ***p < 0.001. Blue points—parameters of the cells cultured with 5% oxygen; green points—parameters of the cells cultured with 21% oxygen.
network structure with thick and branched tubules was observed after 2 and 4 h (Figure 2D).

3.3  |  The influence of the oxygen concentration on long-term adipose stem or stromal cells culture

3.3.1  |  Proliferation impact

The comparative analysis of the cell cultures under different oxygen conditions (5% O₂ and 21% O₂) showed that low physiological oxygen conditions (5% O₂) were associated with a lack of senescence features in cell morphology. However, these morphological changes, for example, loss of spindle shape and strong flattening with granules in the cytoplasm, were strongly expressed in cells cultured with 21% oxygen, especially in the 20th and later passages (Figure 3A).

A higher proliferation potential was observed in ASC culture under physioxic (5% O₂) conditions. This proliferation potential was expressed as significantly higher cell concentrations (cell number/cm²), short PDT and higher CPD in the sixth and later passages (Figure 3B-D), compared with cell culture maintained in 21% O₂.

Additionally, the number of viable cells determined by enzyme-linked assay with WST-1 reagent analysis was significantly higher, confirming the better cell proliferation at a 5% oxygen concentration (Figure 3E).

3.3.2  |  Senescence analysis

The lower efficiency and hardened senescence of ASC cultured with 21% O₂ have been additionally proven by the increase in β-galactosidase reactivity. In the 4th passage, ASCs cultured in 5% O₂ contained 1% of cells with increased expression of β-galactosidase enzyme (ageing cells), while in 21% O₂, the cell population contained 4% of that cell population. This trend was also maintained in later passages. In the 12th passage under physioxic conditions (5% O₂), the subpopulation of senescent ASC was only 4%, whereas in 21% O₂, the subpopulation was 10%. In the 22nd passage with 5% O₂ and 21% O₂, 7% and 96% of the senescent cells showed increased expression of the β-galactosidase enzyme, respectively. The lower oxygen concentration enabled cells (after a long period in culture in vitro) to maintain low β-galactosidase enzyme activity (Figure 3A,F).

3.4  |  The adipose stem or stromal cells influence on fibroblast migration

To investigate the effect of ASC on the rate of wound healing and fibroblast infiltration, ASC and fibroblast cocultures were established. The experiment revealed that ASC settled and shared a niche in an environment dominated by fibroblasts, integrating with them to create a homogenous monolayer (Figure 4A).

3.5  |  Adipose stem or stromal cells cocultured with surgical dressings: Hydrocolloid, gelatin and paraffin

To develop a method of ASC clinical application for small but deep non-healing wounds and ulcerations, an innovative transplant method was tested. ASC were seeded onto commercially available surgical dressings (hydrocolloid, gelatin and paraffin) to prolong the exposure of chronic wounds to the growth factors and immunomodulators secreted by cells and, at the same time, to protect both the transplanted cells and the wound from adverse environmental conditions, i.e., dry air or mechanical trauma. ASC survival on hydrocolloid and paraffin dressings was 77% and 82%, respectively, 24 h after seeding (Figure 5A).

Cell survival was not estimated on gelatin sponges because the dressing showed strong hydrophilic properties and dissolved almost completely after 24 h of incubation. This phenomenon increased the ability of the seeded ASC to form vasculature-like structures (Figure 5B). The same ability to spontaneously create capillary-like structure formation was observed after ASC application to ECMatrix gel. The formation of a mature vascular network structure with thick and branched tubules was observed after 2 and 4 h (Figure 5B).

3.6  |  Irrigator application to extensive wounds

In contrast to small but deep wounds, for extensive, superficial wounds filled with granulated tissue, cells do not require additional scaffolds but must be seeded quickly over a broad area, often several times and preferably non-invasively. For this purpose, we used an irrigator available in the clinic. The experiments determined the application parameters necessary to create the optimal conditions for cells to survive, for example, stream shape, fluid flow rate and medium type. Due to comparable cell survival data (83% and 82.5%) for a focussed or dispersed stream, a surgeon can adapt the dispersion stream to the wound area (Figure 5C). In addition, the rate at which the liquid was dispersed (slow—first mode, intermediate—third mode and fast—fifth mode) did not significantly affect cell survival (82%, 80% and 77% survival, respectively; Figure 5C) and could be adjusted appropriately to wound size (Figure 5C). However, our results strongly indicate that Ringer’s solution is a better liquid than saline for ASC suspensions (0.9% NaCl). The ASC seeded with this irrigator had a high survival rate independent of the tested mode. Notably, the 2000 cells/cm² density used in these experiments in vitro was the limited/minimal
density for cell survival (without inducing an anoikis effect). Therefore, we suggest a similar density for clinical applications in the treatment of non-healing wounds and ulcerations.

3.7 | Clinical cases

After conducting pre-clinical studies, a pilot study of clinical cases was performed. Two clinical cases are presented in Figures 6 and 7.

3.7.1 | Case no. 1

A 70-year-old patient suffered from a chronic wound on the anterior surface of the calf with extensive exposure of the tibial bone. With a diagnosis of advanced atherosclerosis, the patient underwent vascular surgery twice, with angioplasty and stent placement. Due to the ineffectiveness of conservative and surgical treatments of chronic wounds for 7 years, he was referred to the Department of Plastic and Reconstructive Surgery (Figure 6A,B). During treatment, a superficial layer of cortical bone was removed, and cancellous bone was exposed at
the bottom of the wound. Then, SVF solutions (containing ASCs) were prepared according to the described protocol and administered with a spray irrigator to granulated tissue at the edges of the wound and directly on the exposed bone. The wound was covered with a thin skin graft. The patient spent 10 days in the hospital. Gradual and uncomplicated healing processes were observed during the first week.
after surgery, and complete healing was achieved after 4 weeks (Figure 6C). A single procedure was enough to achieve full healing of the wound that had not healed in the previous 7 years. After the 60-month follow-up, no signs of wound recurrence were observed (Figure 6D,E).

3.7.2 Case no. 2

A 60-year-old obese male suffered from a deep, penetrating wound, which remained after a complicated fracture of the proximal tibia and fibula in the early 1980s. The deep, penetrating wound below the...
knee area opened spontaneously 8 years before admission and was resistant to multiple conservative and surgical treatments. The wound was debrided, and an external hydrocolloid dressing containing ASC (from SVF) solution was applied twice at 3-month intervals. Two months after the second treatment, the wound healed completely. At the 36-month follow-up, no signs of wound recurrence were observed (Figure 7).

4 | DISCUSSION

Every human being contains evolutionarily preserved stem cell reservoirs (stem cell niches) that are expected to adapt and improve the quality of the individual's life during acute and chronic diseases. The niches include MSC. A few mechanisms by which MSC influences wound healing promotion can be considered. Cytokine secretion increases the content of growth factors in the wound, modulates PBMC (peripheral blood mononuclear cells) activity, stimulates the proliferation and migration of cells, regenerates damaged epidermis, promotes wound granulation and angiogenesis and remodels the wound structure.\(^{20,21}\) The immunomodulatory function is conditioned by the immune mobilisation and differentiation of MSC stimulated by proinflammatory factors, that is, TNF-α, IL-1β, free radicals, chemokines and leukotrienes that are secreted by, for example, fibroblasts, cells of the immune system or endothelial cells. Immunomobilized MSC leads to the release of growth factors, that is, EGF, FGF, PDGF, TGF-β, VEGF, HGF, IGF-1, Ang-1 and KGF, and anti-inflammatory interleukins, that is, IL-10 or IL-4.\(^{27}\) MSC, although morphologically similar, display tremendous diversity, which is conditioned mainly by their origin—the source tissue. The differences relate not only to the presence/absence of specific surface antigens or the ability to differentiate but also to their paracrine properties.\(^{23}\)

Moreover, the phenomenon of cell plasticity is observed in which, according to the definition, cells adapt to the current needs of the organism.\(^{24}\) This process of conversion of one cell line into another, with the simultaneous loss of markers and functions that are specific for the tissue of origin and the acquisition of new markers and functions that are typical of the target tissue, can be recognised. It has been shown that MSC can differentiate under strictly defined conditions in vitro into myocytes, endothelial cells or cardiomyocytes.\(^{18,25,26}\)

The availability and abundance of MSC, which are a natural pancreas, varies from person-to-person and depends on the location of the disease. According to Gimble et al., the clinical use of stem cells requires choosing a source that ensures that a large number of the derived cells are successfully transplanted with minimal invasiveness.\(^{27}\) These criteria establish adipose tissue as a leader over other sources of stem cells.

In addition, stem cells isolated from adipose tissue and other somatic stem cells do not carry a proven risk of tumorigenesis after transplantation and do not raise ethical controversy.\(^{28-31}\) Additionally, the availability of ASC from autogenic or allogenic sources is problem-free. Annually, one-half million liposuction procedures are carried out in the United States, and 100 to 3000 ml of lipoaspirate is obtained.\(^{27}\) According to the studies of Fraser and colleagues, 2500-fold more MSC are isolated from adipose tissue than from the same amount of bone marrow.\(^{19}\) After optimal source selection, the number of stem cells in the heterogeneous stromal cell population is essential. Commercial devices for regenerative cell isolation from lipoaspirate enabled us to obtain \(1 \times 10^5\)–25 \(\times 10^4\) regenerative cells/g. From that population, CFU-F cells were plated at 0.3–1.6/100 cells, and the cell viability was 57–93%.\(^{32}\) The enzymatic method modified in our laboratory allowed us to obtain 26–39 \(\times 10^4\) regenerative cells/g with 95–98% viability. The isolated cells met the IFATS criteria.\(^{33,34}\)

The analysed cells morphologically resembled fibroblasts adhering to the plastic surface; expressed CD105, CD73 and CD90 surface markers in the absence of haematopoietic antigens CD11B, CD19, CD45 or HLA-DR; and differentiated into other types of cells derived from the same germ layer, that is, adipocytes, chondrocytes and osteoblasts.\(^{35-39}\) Additionally, the extended criteria for mesenchymal stem cells described by Sandwig were fulfilled, and the number of CFU-F cells in our culture was checked to obtain a ratio of CFU-F cells/plated cells of between 5/100 and 6/100.\(^{39}\)

An important complication of non-healing wounds is their impaired microcirculation. We have shown that ASC isolated and cultured by the method described here have very high vascular potential, creating vascular structures in the short term without additional preconditions. Similar observations were published by the Cao group; however, those authors subjected cells to initial and multiday endothelial cell differentiation.\(^{36}\)

The clinical use of stem cells demands the development of a protocol ensuring the maintenance of ASC with a high initial potential for proliferation and slow senescence. In our experiments, we analysed the influence of different oxygen concentrations (5% physioxia and 21% normoxia) on ASC cultured from passages 1 to 20. During the one-week growth analysis of ASC culture, three typical MSC phases were distinguished, namely, the initial phase, logarithmic growth and the plateau phase, in which cells showed a stabilised growth rate. The long-term growth analysis showed that ASC had a stable growth rate from the 8th to the 15th passage under both analysed oxygen conditions; however, at 5% \(O_2\), proliferation was twice as fast.\(^{40}\)

Similar observations were made with MSC derived from Wharton jelly cultured under physioxic conditions.\(^{41}\) Cell senescence under the examined oxygen conditions in the early, medium and late passages was also analysed.

Our results clearly indicate slower ageing of the population cultured under physioxic conditions (up to 7% of ageing cells). In the final cell passage (22nd) analysed, the ageing difference under both aerobic conditions was 89% (Figure 3F). Ninety-six percent of cells cultured with 21% oxygen showed increased β-galactosidase enzyme activity. The same result (>90% of ageing cells cultured in an atmospheric gas mixture) was described by the Mitterberger group.\(^{42}\) The results clearly indicate the beneficial effect of physioxia (5% \(O_2\)), which led to better cell conditions, faster proliferation and slower ageing. ASC cocultured with fibroblasts obtained from skin biopsy samples showed that ASC can integrate and share the environment without changing their immunomodulatory properties. Because of its adjuvant
properties, ASC in the scratch test triggered quicker fibroblast proliferation and migration. This result indicates a beneficial (stimulating healing) effect on these cells. Similar results demonstrating ASC paracrine effects, increasing the proliferation and migration of normal keratinocytes and fibroblasts in vitro, as well as in vivo, following the administration of ASC directly to wounds were described by Kim’s group.43 These properties of ASC were described for patients with enterovesical fistulae due to Crohn’s disease in clinical trials of autologous adipose-derived regenerative cell-based therapy.44 It is postulated that the described therapeutic effect is also related to the ability of ASC to differentiate toward pericytes, which are capable of stabilising microvessels and to exert paracrine effects enhancing and/or promoting angiogenesis.45–47 In a mouse model of hindlimb ischemia, it has been shown that the administration of adipose tissue stem cells improves blood flow and capillary density.45 Our observations regarding the ability of ASC to spontaneously form capillary-like structures on ECMMatrix gel confirmed the strong vasculogenic characteristics of this cell source. The same phenomenon was observed after cell administration on a hemostatic gelatin sponge. These results indicate the strong vasculogenic properties of skin-grafted ASC, critical for supporting wound healing. Consistent with the abovementioned results and the literature, the beneficial effect of the epidermal administration of mesenchymal stem cells in the treatment of non-healing wounds has been confirmed in vitro. To introduce the results in the clinic, we attempted to develop simple methods for ASC application. Jia's group compared two methods of cell application: subcutaneous injection or covering the wound with a silicon dressing containing cells. They observed both cell migration within the wound and wound healing, which was not observed in the case of Ringer’s solution- or dressing only-treated wounds.1 For simple application only in the clinic, we decided to examine commercial dressings: paraffin and hydrocolloid. The results confirm good cell survival (80%) inside these dressings, which can enhance the delivery of cells to the wound surface. Subcutaneous administration may be harmful when several dozen injections must be performed and when anaesthesia is required. The method of superficial administration with irrigators may be an alternative treatment. Proposed by our group, the optimal cell seeding density is 2000–3000 cells/cm² (which is the optimal density for cell survival in vitro) on the wound surface, and the recommended fluid for cell suspension and administration is Ringer’s solution. Cells infused with the irrigator under fluid pressure are implanted into the wound granulation tissue/parenchyma, which allows them to easily integrate and prevents them from flowing out of the wound edges. When assessing the type of stream that can be used, no significant differences were found for these cells; therefore, a concentrated or diffuse stream of the irrigator can be selected by the surgeon for each individual case, taking into account the extent of the wound. Similarly, the rate of administration (flow rates from 1 to 5) did not generate significant differences in cell survival after application to the wound; therefore, it can be arbitrarily modified, but (it should be underlined) the smallest volume of buffer with cells for irrigator use in our test was 20 ml—for smaller volumes, it was difficult to disperse with the use of this equipment. The ability to provide ASC with our repetitive methods (irrigator or dressing), as well as the immunomodulatory and vasculogenic properties of the final cell population, provides the basis for the development of the personalised treatment of chronic wounds. To date, we do not have methods for cell labelling for detecting their migration and fate in humans, making it difficult to show and explain the mechanisms that are critical for their therapeutic action. However, as presented in this article, the clinical results of the treatment of individual patients (Figures 6 and 7) are deeply encouraging.

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CONFLICT OF INTEREST
The authors declare that there are no competing financial interests or conflicts of interest regarding the publication of this paper.

DATA AVAILABILITY STATEMENT
The data that support the findings of this study are available on request from the corresponding author. The data are not publicly available due to privacy or ethical restrictions.

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