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ORIGINAL RESEARCH-BASIC SCIENCE

Wound Repair and Regeneration — WILEY

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.[1] 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.[2] 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.[3]

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.[4] Both stem cell populations, BMSC and ASC, are of mesenchymal origin, but their adjuvant properties differ.[5–7]

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.[8]

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.[9,10] However, intratissue oxygen concentrations differ from 2% to 9% O_2 (14–65 mm Hg). This range has been recognised as physiological normoxia, called physioxia.[9,11] 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.[9–17]

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 lipoaspirate 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.

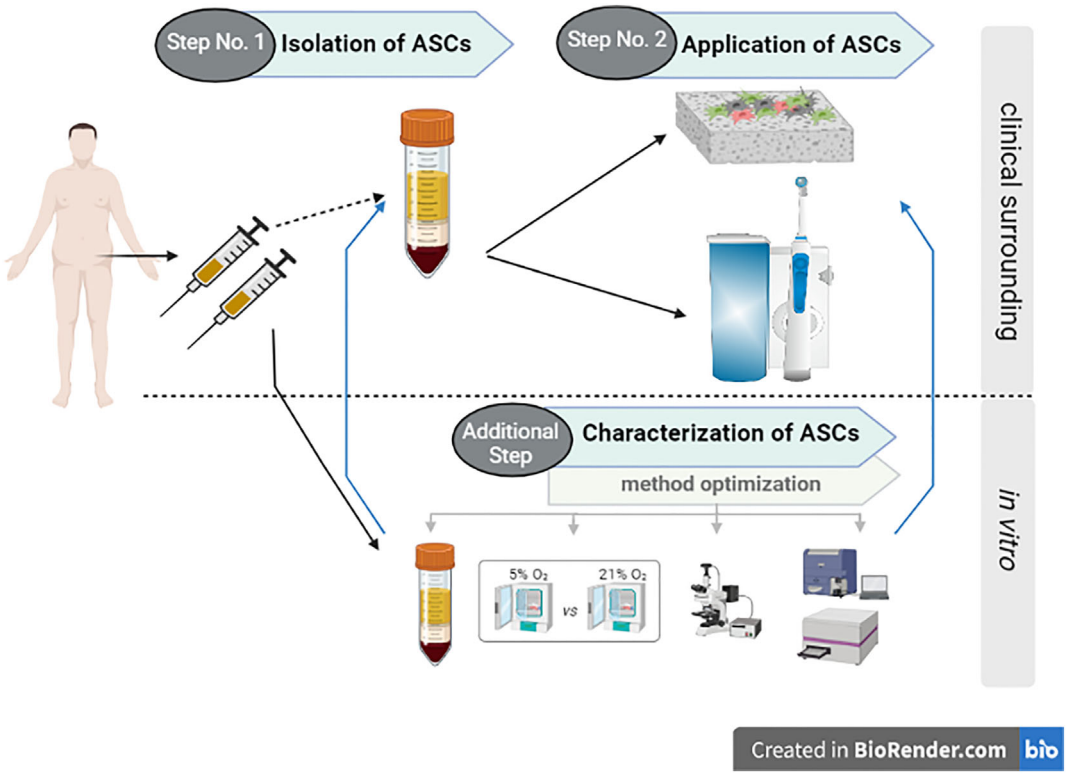

FIGURE 1 Scheme of the experiments carried out. The first step of our study was to optimise the method of ASC isolation for easy implementation in the clinical procedure. Additionally, the aim of our study was to optimise cell culture conditions (based on the analysis of ASC with the application of microscopic and cytometric techniques as well as microplate tests) and methods for cell delivery into wounds while maintaining their high survival, proliferation and colonisation potential

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% CO_2 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 antimycotic, 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% O_2 and 5% CO_2. 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 5×10^3 cells/cm^2. 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 1×10^6 cells/ml were suspended in a cold buffer designed for flow cytometry (amplifying the fluorescence signal) with BD Pharmingen. 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 FACSDiva software. A total of 1×10^5 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: 1×10^4 cells/cm^2; for the osteogenesis experiments: 5×10^3 cells/cm^2; and for the chondrogenesis experiments: 1.6×10^3 cells/cm^2) and cultured to subconfluency in MSC growth medium (Lonza) under 21% O_2.

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 1000*g* for 3 min. Subsequently, the cells were seeded on Matrigel at a density of 5×10^4 cells/cm^2 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/cm^2. 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.[18] Briefly, proliferation parameters were calculated based on the total cell number at each passage using the following formulas: $PDT = (t - t0) \times \log 2/(\log N - \log N0)$ and $CPD = PDs_1 + PDs_2 + PDs_3 + \ldots + PDsn$, where $PDs = \log(N0/N)/\log 2$. The duration of passage, counted in days, is equal to t – t0. *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 2 h. To determine the proportion of viable cells, the reduction in the level of tetrazolium salt-induced by mitochondrial dehydrogenases with respect to the level of soluble formazan was analysed 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 senescent 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 3×10^3 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 semiconfluency was obtained. Then, the ASC labelled with CMFDA were seeded on the fibroblast layer at a density of 10^3 cells/cm^2. ASC with fibroblasts were cocultured in MSCG 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 2×10^5 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 (~ 9.6×10^5 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.[19] 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).

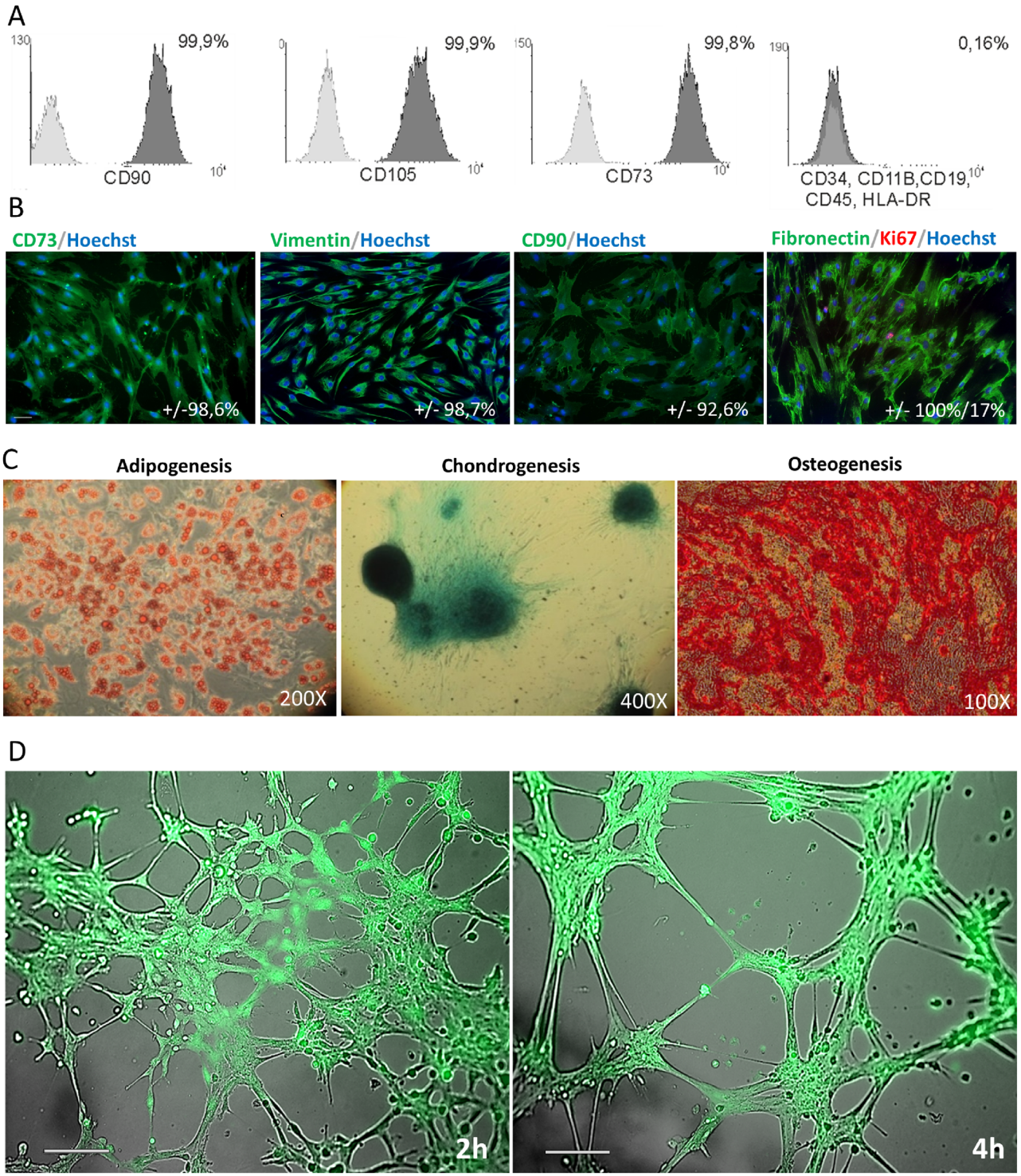

FIGURE 2 Characterisation of the ASC. (A) Flow cytometry analysis showed typical and relatively high expression of specific mesenchymal markers (CD73, CD90 and CD105). Similarly, less than 1% of the ASC expressed negative markers (CD34, CD11b, CD19, CD45 or HLA-DR). (B) Immunocytochemical analysis of 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

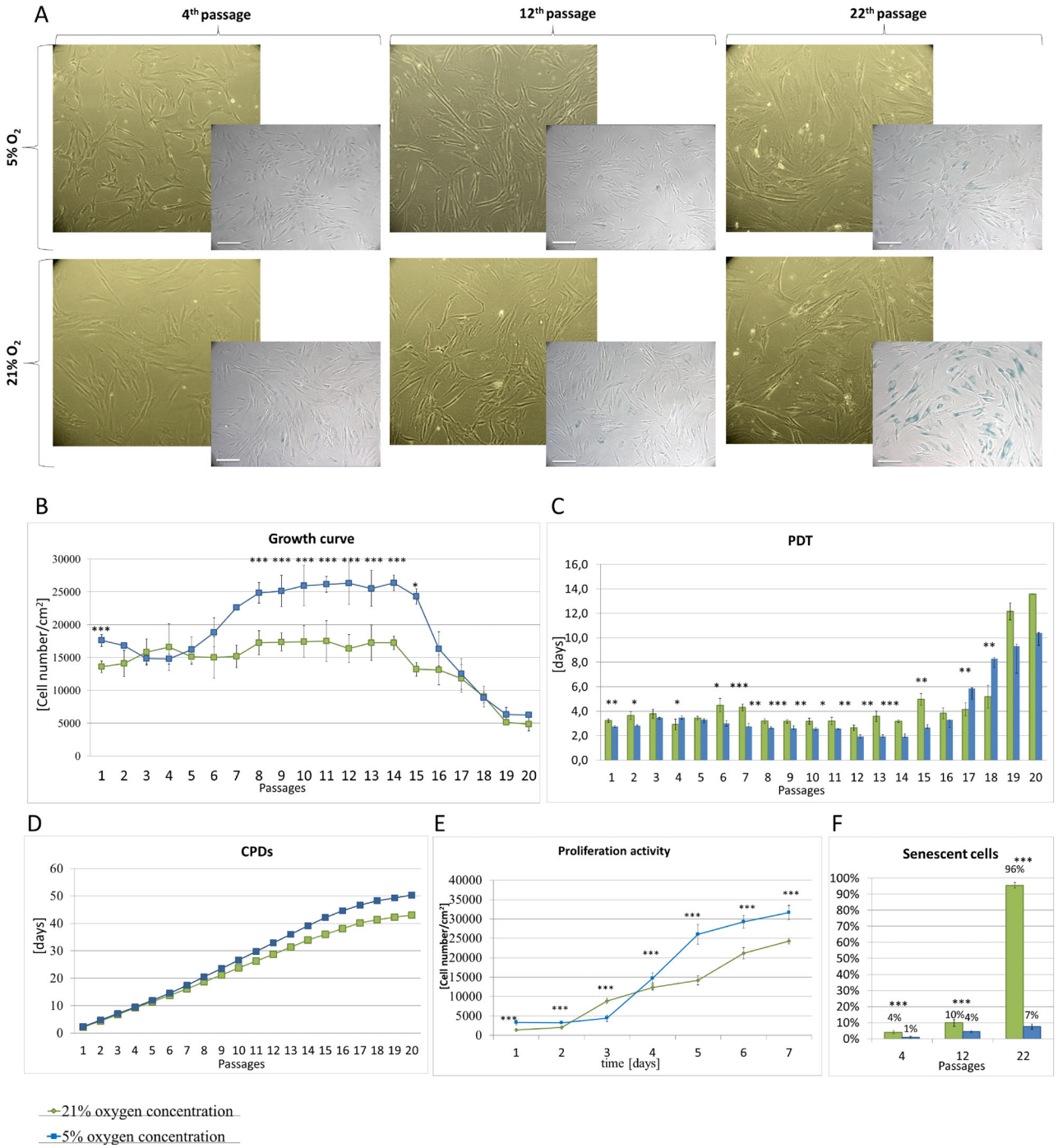

FIGURE 3 Proliferation and senescence analysis of ASC cultured under 5% and 21% O_2 conditions. (A) Morphological and SA-β-gal activity changes in ASC during long-term culture. Photos magnified 100×. Scale bar: 200 μm. (B) Growth curve of the ASC. ASC cultured with 5% O_2 proliferated faster. The results showed significantly higher cell numbers per cm^2 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% O_2 and 21% O_2) showed that low physiological oxygen conditions (5% O_2) 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% O_2) conditions. This proliferation potential was expressed as significantly higher cell concentrations (cell number/cm^2), short PDT and higher CPD in the sixth and later passages (Figure 3B–D), compared with cell culture maintained in 21% O_2.

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 hastened senescence of ASC cultured with 21% O_2 have been additionally proven by the increase in β-galactosidase reactivity. In the 4th passage, ASCs cultured in 5% O_2 contained 1% of cells with increased expression of β-galactosidase enzyme (ageing cells), while in 21% O_2, 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% O_2), the subpopulation of senescent ASC was only 4%, whereas in 21% O_2, the subpopulation was 10%. In the 22nd passage with 5% O_2 and 21% O_2, 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).

To demonstrate the capacity of ASC to enhance the therapy of non-healing wounds, a scratch assay was performed with a fibroblast culture (control group) and coculture of fibroblasts and ASC. The results (after 24 and 48 h) of this experiment demonstrate that coculturing ASC with fibroblasts stimulated the migration and proliferation of the fibroblasts (after 24 h—there were more than 7 cells on the scratch area, which significantly increased after 48 h to more than ~29 cells on the scratch area) (Figure 4B).

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/cm^2 density used in these experiments in vitro was the limited/minimal

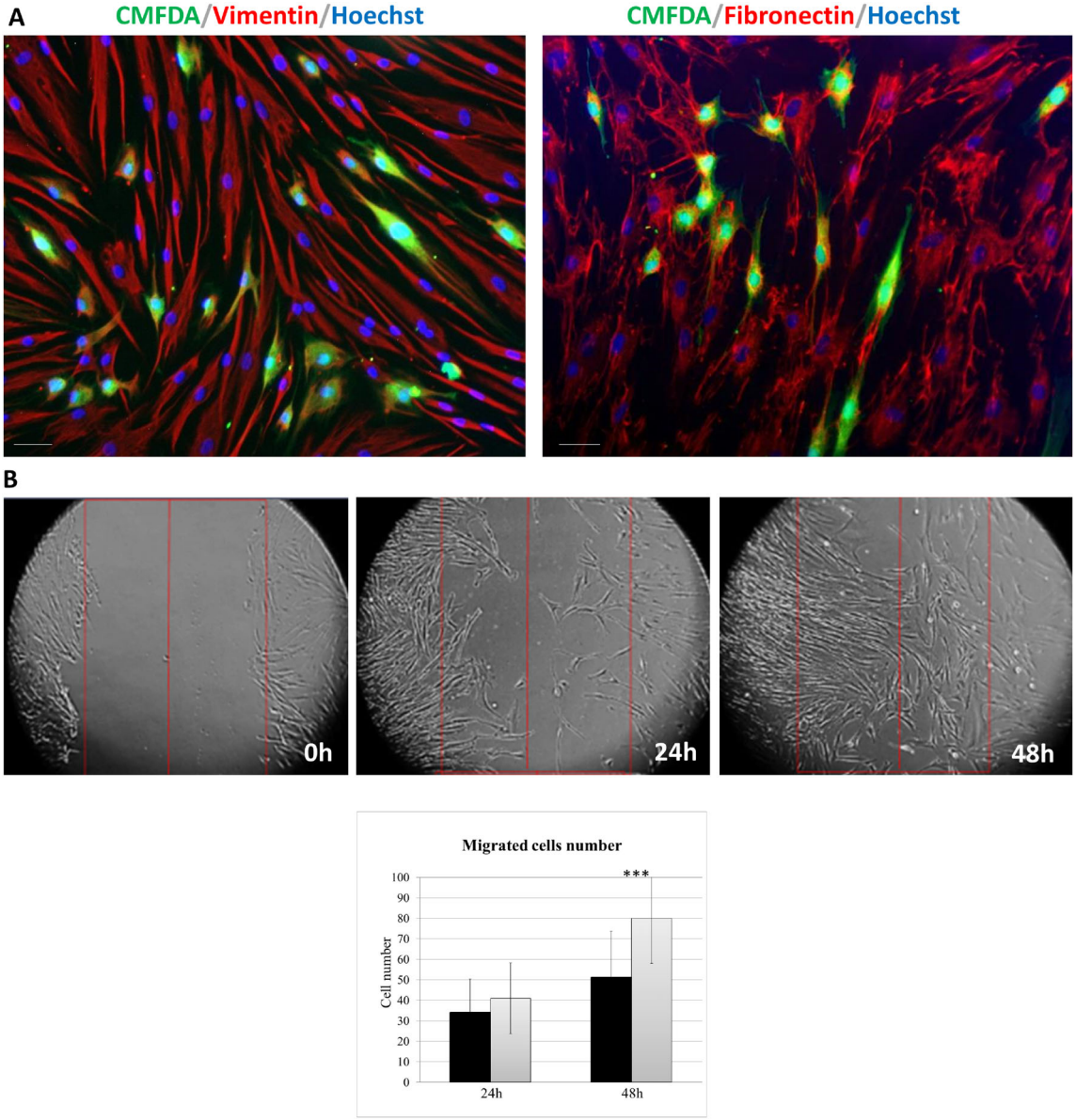

FIGURE 4 Characterisation of the ASC and fibroblast coculture. (A) ASC and fibroblast integration analysis. Good integration, possible niche sharing and a common monolayer of ASC and fibroblasts were visualised, with vimentin and fibronectin labelling shown in red, ASC shown by the green CMFDA tracer, and the cell nuclei stained blue with Hoechst stain. Scale bar: 50 μm. (B) The effect of ASC on fibroblast migration—scratch assay. Photos show the migratory properties of cocultured ASC and fibroblasts at three different time points—0, 24 and 48 h at a magnification of 100×. The graph shows the migratory properties of fibroblasts compared with fibroblasts cocultured with ASC. Fibroblasts cocultured with ASC presented a faster overgrowth rate, especially after 48 h. The results are presented as the mean ± SD of at least 4 experiments; $^{***}p < 0.001$

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

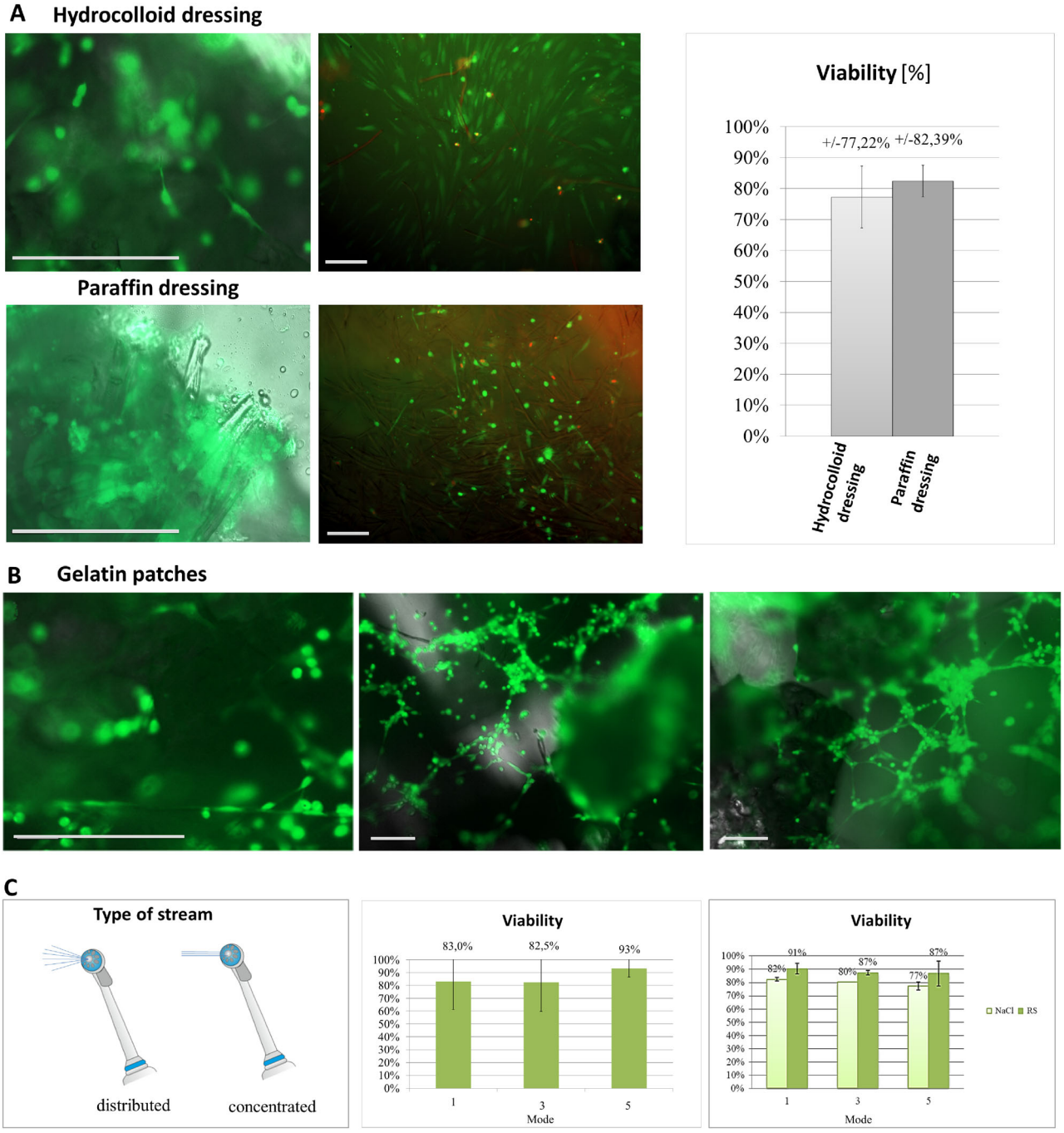

FIGURE 5 Optimization of the methods for ASC wound application. (A) ASC cocultured with hydrocolloid and paraffin dressings. Photos show the CMFDA tracer-labelled ASC in coculture with hydrocolloid and paraffin patches and live/dead staining with live cells labelled with calcein (green) and dead cells labelled with the ethidium homodimer (red). High survival (±77% and ±82%) of these cells 24 h after seeding on hydrocolloid and paraffin patch surfaces was proven. (B) Analysis of the vasculogenic potential of ASC seeded on gelatin patches. ASC formed a network, and pictures were taken at three time points: 0 h (the first image presents ASC cocultured with a gelatin sponge before application on Matrigel), 2 h (second image) and 4 h (third image) after seeding of the ASC on Matrigel. Scale bar: 100 μM. (C) Irrigator application to cover extensive wounds. Comparable ASC survival was confirmed for concentrated and distributed streams: ±83% and ±82.5%. The rate of fluid delivery (slow—1st mode, intermediate—3rd mode and fast—5th mode) did not influence ASC survival during wound application. Suspending ASCs in Ringer's solution resulted in greater cell viability than suspending ASCs in sodium chloride (0.9% NaCl) in the three different speed modes of liquid delivery. Scale bar: 100 μM

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

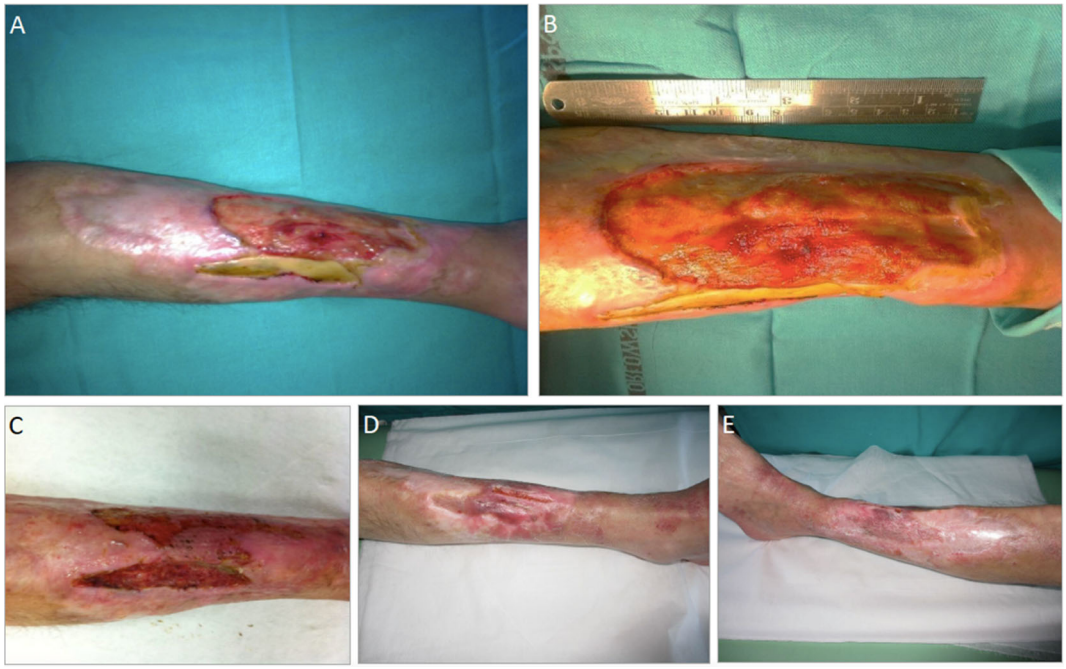

FIGURE 6 Clinical ASC application to an extensive wound. (A and B) A 70-year-old patient with a seven-year history of arteriosclerosis with a chronic wound on the lower leg with tibial bone exposure after vascular angioplasties in both legs and failed attempts with conservative and surgical wound treatments. (C) Two weeks after cortical bone layer removal, ASC were administered with the irrigator and a wound were covered with split thickness skin graft. Total wound closure was achieved in 4 weeks. (D and E) Sixty-month follow-up; no signs of wound recurrence

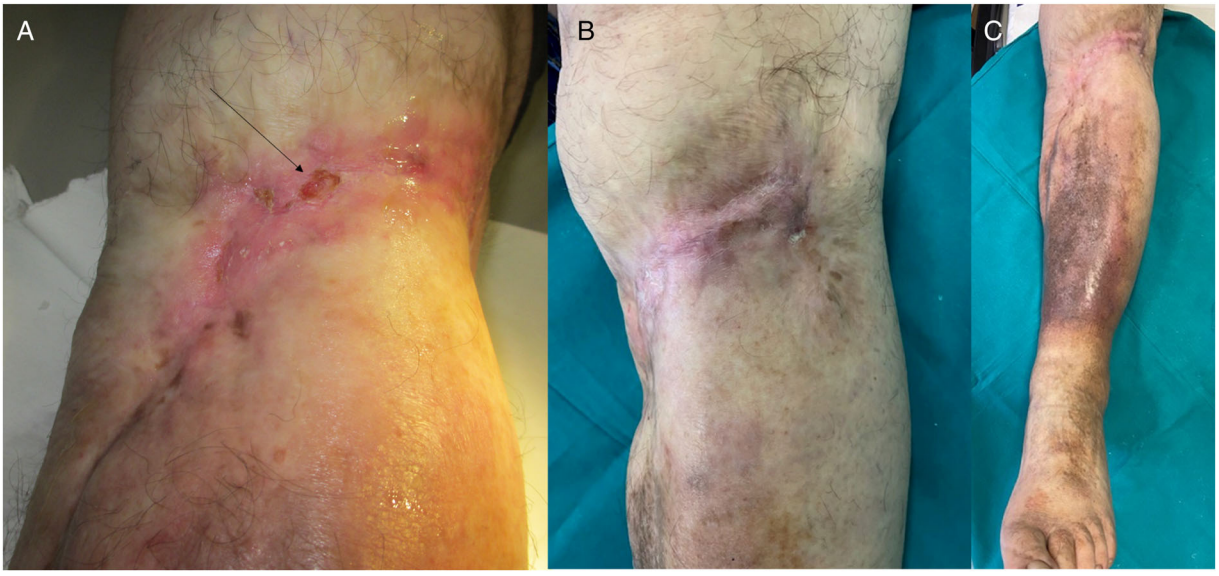

FIGURE 7 Clinical ASC application to a deep, penetrating wound. (A and B) A deep and penetrating wound after complicated fracture of the proximal tibia and fibula in a 60-year-old obese man. Two applications of ASC with dressing were performed. (C) At the 36-month follow-up, no sign of wound recurrence was observed

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.[22] 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 panacea, 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 1×10^5–25×10^4 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.[35–38] 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 ECMatrix 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. Jianga'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/cm^2 (which is the optimal density for cell survival in in vitro tests) 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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REFERENCES

1. Jiang D, Qi Y, Walker NG, et al. The effect of adipose tissue derived MSCs delivered by a chemically defined carrier on full-thickness cutaneous wound healing. *Biomaterials*. 2013;34(10):2501-2515.
2. Harding KG, Morris HL, Patel GK. Science, medicine and the future: healing chronic wounds. *BMJ*. 2002;324(7330):160-163.
3. Melief SM, Zwaginga JJ, Fibbe WE, Roelofs H. Adipose tissue-derived multipotent stromal cells have a higher immunomodulatory capacity than their bone marrow-derived counterparts. *Stem Cells Transl Med*. 2013;2(6):455-463.
4. Wilson WR, Hay MP. Targeting hypoxia in cancer therapy. *Nat Rev Cancer*. 2011;11(6):393-410.
5. Fischer LJ, McIlhenny S, Tulenko T, et al. Endothelial differentiation of adipose-derived stem cells: effects of endothelial cell growth supplement and shear force. *J Surg Res*. 2008;152(1):157-166.
6. Baer PC, Brzoska M, Geiger H. Epithelial differentiation of human adipose-derived stem cells. *Methods Mol Biol*. 2011;702:289-298.
7. Harris LJ, Abdollahi H, Zhang P, McIlhenny S, Tulenko T, DiMuzio PJ. Differentiation of adult stem cells into smooth muscle for vascular tissue engineering. *J Surg Res*. 2012;168(2):306-314.
8. Kim DS, Ko YJ, Lee MW, et al. Effect of low oxygen tension on the biological characteristics of human bone marrow mesenchymal stem cells. *Cell Stress Chaperones*. 2016;21:1089-1099.
9. Mohyeldin A, Garzon-Muvdi T, Quiñones-Hinojosa A. Oxygen in stem cell biology: a critical component of the stem cell niche. *Cell Stem Cell*. 2010;7(2):150-161.
10. Shooter RA, Gey GO. Studies of the mineral requirements of mammalian cells. *Br J Exp Pathol*. 1952;33:98-103.
11. Simon MC, Keith B. The role of oxygen availability in embryonic development and stem cell function. *Nat Rev Mol Cell Biol*. 2008;9:285-296.
12. Wang DW, Fermor B, Gimble JM, Awad HA, Guilak F. Influence of oxygen on the proliferation and metabolism of adipose derived adult stem cells. *J Cell Physiol*. 2005;204:184-191.

13. Xu P, Malladi M, Chiou E, Bekerman AJ, Giaccia MT. *In vitro* expansion of adipose-derived adult stromal cells in hypoxia enhances early chondrogenesis. *Tissue Eng*. 2007;13:2981-2993.
14. Malladi P, Xu Y, Chiou M, Giaccia AJ, Longaker MT. Effect of reduced oxygen tension on chondrogenesis and osteogenesis in adipose-derived mesenchymal cells. *Am J Physiol Cell Physiol*. 2006;290: C1139-C1146.
15. Annabi B, Lee YT, Turcotte S, et al. Hypoxia promotes murine bone-marrow-derived stromal cell migration and tube formation. *Stem Cells*. 2003;21:337-347.
16. Rosova I, Dao M, Capoccia B, Link D, Nolta JA. Hypoxic preconditioning results in increased motility and improved therapeutic potential of human mesenchymal stem cells. *Stem Cells*. 2008;26: 2173-2182.
17. Lee CT, Mace T, Repasky EA. Hypoxia-driven immunosuppression: a new reason to use thermal therapy in the treatment of cancer. *Int J Hyperthermia*. 2010;26(3):232-246.
18. Chamberlain G, Fox J, Ashton B, Middleton J. Concise review: mesenchymal stem cells: their phenotype, differentiation capacity, immunological features, and potential for homing. *Stem Cells*. 2007;25(11): 2739-2749.
19. Fraser JK, Zhu M, Wulur I, Alfosno Z. Adipose-derived stem cells. mesenchymal stem cells. *Methods Mol Biol*. 2008;449:59-67.
20. Ebrahimian TG, Pouzoulet F, Squiban C, et al. Cell therapy based on adipose tissue derived stromal cells promotes physiological and pathological wound healing. *Arterioscler Thromb Vasc Biol*. 2009;29: 503-510.
21. Cherubino M, Rubin JP, Miljkovic N, Kelmendi-Doko A, Marra KG. Adipose-derived stem cells for wound healing applications. *Ann Plast Surg*. 2011;66(2):210-215.
22. Ma S, Xie S, Li W, Yuan B, Shi Y. Immunobiology of mesenchymal stem cells. *Cell Death Differ*. 2014;21:216-225.
23. Petrenko Y, Vackova I, Kekulova K, et al. A comparative analysis of multipotent mesenchymal stromal cells derived from different sources, with a focus on neuroregenerative potential. *Sci Rep*. 2020; 10:4290.
24. Wagers AJ, Weismann IL. Plasticity of adult stem cells. *Cell*. 2004; 116(5):639-648.
25. Blau HM, Brazelton TR, Weimann JM. The evolving concept of a stem cell: entity or function? *Cell*. 2001;105(7):829-841.
26. Williams AR, Hare JM. Mesenchymal stem cells: biology, pathophysiology, translational findings, and therapeutic implications for cardiac disease. *Circ Res*. 2011;109(8):923-940.
27. Gimble JM, Katz AJ, Bunnell BA. Adipose-derived stem cells for regenerative medicine. *Circ Res*. 2007;100(9):1249-1260.
28. Lech W, Figiel-Dabrowska A, Sarnowska A, et al. Phenotypic, functional, and safety control at preimplantation phase of MSC-based therapy. *Stem Cells Int*. 2016;2016:2514917-2514913.
29. Bajek A, Olkowska J, Drewa T. Mesenchymal stem cells as a therapeutic tool in tissue and organ regeneration. *Postępy Hig Med Dosw*. 2011;65:124-132. Polish.
30. Domanska-Janik K. Zarodkowe vs somatyczne komorki macierzyste w terapii regeneracyjnej mozgu. *Postepy Biol Kom*. 2005; 32(Suppl 23) 27–40. Polish.
31. Zielinska E. Dopuszczalnosc wykorzystania zarodkowych komorek macierzystych do celow badawczych. *Postepy Biol Kom*. 2005;32(23): 5-10. Polish.
32. Aronowitz JA, Ellenhorn JD. Adipose stromal vascular fraction isolation: a head-to-head comparison of four commercial cell separation systems. *Plast Reconstr Surg*. 2013;132(6):932-939.
33. Bourin P, Bunnell BA, Casteilla L, et al. Stromal cells from the adipose tissue-derived stromal vascular fraction and culture expanded adipose tissue-derived stromal/stem cells: a joint statement of the International Federation for Adipose Therapeutics and Science (IFATS) and the International Society for Cellular Therapy (ISCT). *Cytotherapy*. 2013;15(6):641-648.
34. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. *Cytotherapy*. 2006;8(4):315-317.
35. Riekstina U, Cakstina I, Parfejevs V, Hoogduijn M. Embryonic stem cell marker expression pattern in human mesenchymal stem cells derived from bone marrow, adipose tissue, heart and dermis. *Stem Cell Rev*. 2009;5(4):378-386.
36. Cao Y, Sun Z, Liao L, Meng Y, Han Q, Zhao RC. Human adipose tissue-derived stem cells differentiate into endothelial cells *in vitro* and improve postnatal neovascularization *in vivo*. *Biochem Biophys Res Commun*. 2005;332:370-379.
37. Zuk PA, Zhu M, Mizuno H, et al. Multilineage cells from human adipose tissue: implications for cell-based therapies. *Tissue Eng*. 2001;7:211-228.
38. Rodriguez AM, Pisani D, Dechesne CA, et al. Transplantation of a multipotent cell population from human adipose tissue induces dystrophin expression in the immunocompetent mdx mouse. *J Exp Med*. 2005;201(9):1397-1405.
39. Sandvig I, Gadjanski I, Vlaski-Lafarge M, et al. Strategies to enhance implantation and survival of stem cells after their injection in ischemic neural tissue. *Stem Cells Dev*. 2017;26(8):554-565.
40. Christodoulou I, Kolisis FN, Papaevangeliou D, Zoumpourlis V. Comparative evaluation of human mesenchymal stem cells of fetal (Wharton's jelly) and adult (adipose tissue) origin during prolonged *in vitro* expansion: considerations for cytotherapy. *Stem Cells Int*. 2013; 2013:246134, 1–12.
41. Drela K, Sarnowska A, Siedlecka P, et al. Low oxygen atmosphere facilitates proliferation and maintains undifferentiated state of umbilical cord mesenchymal stem cells in an hypoxia inducible factor-dependent manner. *Cytotherapy*. 2014;16(7):881-892.
42. Mitterberger MC, Zwerschke W. Mechanisms of resveratrol-induced inhibition of clonal expansion and terminal adipogenic differentiation in 3T3-L1 preadipocytes. *J Gerontol A Biol Sci Med Sci*. 2013;68(11): 1356-1376.
43. Kim W-S, Park B-S, Park S-H, Kim H-K, Sung J-H. Antiwrinkle effect of adipose-derived stem cell: activation of dermal fibroblast by secretory factors. *J Dermatol Sci*. 2009;53(2):96-102.
44. Moniuszko A, Sarnowska A, Rogowski W, Durlik M, Wluka A, Rydzewska G. Successful treatment of an enterovesical fistula due to Crohn's disease with stem cell transplantation: a case report. *Prz Gastroenterol*. 2018;13(4):332-336.
45. Nakagami H, Morishita R, Maeda K, et al. Adipose tissue-derived stromal cells as a novel option for regenerative cell therapy. *J Atheroscler Thromb*. 2006;13(2):77-81.
46. Amos PJ, Mulvey CL, Seaman SA, et al. Hypoxic culture and *in vivo* inflammatory environments affect the assumption of pericyte characteristics by human adipose and bone marrow progenitor cells. *Am J Physiol Cell Physiol*. 2011;301(6):1378-1388.
47. Traktuev DO, Merfeld-Clauss S, Li J, et al. A population of multipotent CD34-positive adipose stromal cells share pericyte and mesenchymal surface markers, reside in a periendothelial location, and stabilize endothelial networks. *Circ Res*. 2008;102(1):77-85.

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