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ORIGINAL ARTICLE

Autotransplantation of the Adipose Tissue-Derived Mesenchymal Stromal Cells in Therapy of Venous Stasis Ulcers

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Abstract

Adipose tissue is a reliable source of mesenchymal stromal cells (MSC) for use in regenerative medicine. The aim of this pilot study was to describe the method, and assess the safety and the potential efficacy of transplantation of autologous adipose tissue-derived MSC for the treatment of chronic venous stasis ulcers. Study group consisted of 11 patients (mean age: 66.6 ± 9.5 years) with chronic venous stasis ulcers. Adipose tissue was harvested by tumescent-aspiration method. Stromal cells were separated using a dedicated closed system in a real-time bedside manner. The phenotype of cells was determined immediately after separation. Cell concentrate was implanted subcutaneously around the wound and the wound bed. All ulcers were assessed planimetrically before autotransplantation and every two weeks during the six-month follow-up. During the study all patients received standard local and general treatment. The preparation contained an average of $5.6 \times 10^6 \pm 4 \times 10^6$ cells per milliliter. The phenotype of 65–82% of transplanted cells expressed MSC markers: $CD73^+$ $CD90^+$ and $CD34^+$. An improvement was observed in 75% of ulcers. The data showed highly significant negative correlation ($p < 0.0001$) between wound size and wound closure degree. There was no correlation of ulcer healing with other parameters evaluated, including age of the patients. No serious side effects were observed. Autotransplantation of adipose tissue stromal cells may be a safe and promising treatment method for chronic venous ulcers.

Keywords Adipose tissue-derived mesenchymal stromal cells · CELLUTION 800 · Regenerative medicine · Wound healing · Venous stasis ulcers

Introduction

Venous leg ulcers (VLUs), the extreme stage of chronic venous insufficiency, are the main cause of chronic wounds (70–90%). Treatment requires cooperation of various specialists, which is burdensome for the patient and expensive (Jawień et al. 2011; Rybak 2003).

The latest guidelines indicate that such wounds should initially be treated in accordance with standard practice and guidelines for wound care, the TIME Framework (Leaper et al. 2012; Schultz et al. 2003). Patients who do not achieve 50% wound area reduction are recommended for further therapy using advanced regenerative medicine, including stromal cells (Nuschke 2014; Otero-Viñas and Falanga 2016). There has been significant progress in research on stromal cells and their use as an effective tool in biological therapy of many diseases (Bajek et al. 2011; Gimble et al. 2007; Sarvajnamurthy et al. 2003). Stromal cells are used to treat patients with hematological, oncological, dermatological, ophthalmic and orthopedic diseases, and the scope of possible applications goes beyond the idea of using them only as spare parts (Sarvajnamurthy et al. 2003).

Mesenchymal stromal cells (MSCs) are defined according to the criteria of the International Society for Cellular Therapy as adherent stromal cells with the phenotype $CD73^+$, $CD90^+$, $CD105^+$, $CD45^-$ and $HLA\text{-}DR^-$, capable of differentiating into at least three cell types: osteoblasts, chondroblasts and adipocytes (Pittenger et al. 1999). In the literature, these cells are also often referred to as ADSC

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(adipose stem cells), SVF (stromal vascular fraction) or MAPC (multipotent adult progenitor cells), each of which describes a different aspect of these not explicitly defined cells. MSC are isolated from various tissues, including bone marrow, peripheral blood, umbilical cord blood, skin, muscles, adipose tissue and synovial membrane. An increasingly popular source of MSC is adipose tissue (Li et al. 2018; Zuk et al. 2002). It is an excellent source of stromal cells because of its ease of collecting and minimal discomfort experienced by the patient during the liposuction procedure.

MSCs have been shown to play an important role in wound healing, mainly by means of producing biologically active agents that promote proliferation and migration of endothelial cells, fibroblasts and skin cells (López et al. 2018; Maxson et al. 2012). Research also shows that the ability to modulate activity of the immune system may be due to the positive effects these cells cause. Apart from the pro-angiogenic, chemoattractive and anti-inflammatory potential of the factors secreted by MSC, there are also factors with anti-apoptotic, antibacterial or anti-fibrotic activity that may also affect the healing process of wounds (Otero-Viñas and Falanga 2016; Strioga et al. 2012).

These characteristics have led to attempts to use MSCs in the treatment of difficult to heal chronic wounds (Otero-Viñas and Falanga 2016). Very good therapeutic effects were obtained using allogeneic MSCs derived from porcine adipose tissue, where a significant reduction in wound surface was observed after applying the cells in a dose-dependent manner (James et al. 2018). Reduction of burn wounds after the application of autologous stem cells isolated from adipose tissue was also demonstrated in Wistar rats, which additionally showed a faster healing process by applying an injection around the bottom of the wound compared to when administering the cells directly to the bottom of the wound, as well as in comparison with the allogeneic transplantation of cells (Chang et al. 2018). So far, the clinical utility of MSC has been evaluated in dermatology, cardiology, autoimmune diseases, neurology and orthopedics, as well as in angiology in the treatment of peripheral vascular diseases (Kesten and Fraser 2016; Lu et al. 2011; Teraa et al. 2013). In a pilot human study, autologous, uncultured adipose-derived SVF cells were applied to treat patients with critical limb ischemia. Clinical improvement was observed in 86.7% of the patients, probably due to the formation of numerous vascular collateral networks (Darinskas et al. 2017).

The aim of this pilot study was to assess the safety and clinical efficacy of the applied autologous transplantation procedure, using non-expanded mesenchymal stromal cells isolated from adipose tissue (AT-MSC) in the treatment of chronic and difficult to heal venous ulcers. Particular attention was paid to the size of the wound and the effect of using these cells, assuming that the therapeutic effect may depend to a large extent on the size of the wound.

Materials and Methods

Patients

The study group consisted of 11 patients with chronic venous ulcers: 5 men and 6 women. The average age of patients was 66.6 ± 9.5 years. Twelve ulcers were treated (one patient with two wounds on both lower limbs). The size of ulcers ranged from 7.7–147.0 cm^2 (mean: 53.9 cm^2).

Details of patient characteristics are presented in Table 1.

Criteria for including patients in the study included:

- age over 18 years old;

Table 1 Characteristics of patients with venous leg ulcers

No.	Sex	Age	Cholesterol (mg/dL)	HDL (mg/dL)	Triglycerides (mg/dL)	LDL (mg/dL)	CRP (mg/L)	Glucose (mg/dL)	BMI
1	F	74	203	46	130	131	4.5	92	66.20
2	M	62	210	33	266	124	2.5	113	25.00
3	F	68	187	53	82	118	2.1	79	24.22
4	M	85	137	28	59	97	9.3	89	33.96
5	M	56	250	61	131	163	5.7	96	36.75
6	F	74	156	44	116	89	0.5	70	47.50
7	F	57	227	43	138	156	10.0	102	40.64
8	F	69	202	56	141	118	6.3	139	35.94
9	M	82	114	34	194	230	7.4	93	33.96
10	M	64	97	48	199	269	7.6	112	37.18
11	F	66	101	53	170	78	4.4	87	34.11
11	F	66	101	53	170	78	4.4	87	34.11

CRP C-reactive protein, *BMI* body mass index, *HDL* high-density lipoprotein, *LDL* low-density lipoprotein

- diagnosed venous insufficiency, confirmed in each case by a positive ultrasound examination, USG-duplex Doppler scan;
- location of ulcers on the lower leg above the ankle, either lateral or medial;
- surface of the ulcer > 3 cm^2;
- duration of the disease for more than six weeks with inefficient previous standard treatment.

Exclusion criteria included:

- use of anticoagulants, immunosuppressants or general steroid therapy (during the course of the study);
- active or past tumor;
- ankle brachial index value above 0.9;
- C-reactive protein level above 10 mg/L;
- symptoms of a clinically active infection in or around the wound;
- pre-menopausal period in women;
- hypersensitivity to lidocaine.

Patients were treated with standard of care before inclusion to the study. Standard of care was based on TIME strategy. This standard included tissue debridement, infection and inflammation control, moisture balance maintaining and epidermization stimulation. Additionally, subjects were treated for primary disease contributing to the lack of wound healing.

Ethical Statement

The research was carried out as part of the Wrovasc project. The study protocol was approved by the local bioethics committee at the Regional Specialist Hospital, Research and Development Center in Wroclaw (No. KB/27/2015); all procedures performed in the study were carried out in accordance with accepted ethical standards contained in the Declaration of Helsinki 1975 (as revised in 2000). All patients were informed prior, in detail, about the purposes and methods involved in the study, as well as the potential benefits and risks of the therapy. All subjects expressed their informed consent in writing to participate in the study.

Stromal Cell Isolation

Adipose tissue was collected once from the patients' abdominal area in the amount of 360 mL by the tumescent-aspiration method after infiltration with isotonic fluid, under local anesthesia of the abdominal integuments with 1% lidocaine solution. Stromal cells were isolated from the lipoaspirate, with a closed and fully automated CELLUTION 800 system (Cytori Therapeutics, USA), based on enzymatic preparation using Celase as the processing enzyme. As a result of the separation process, 5 mL of cell suspension was obtained from each tissue sample every time. The resulting patient's own suspension of cells in the volume of 4.5 mL was administered once to the tissues surrounding the ulcers and under the ulcer bed by repeated injections (0.25–0.5 mL cell suspension per injection) after cleansing the wound according to the TIME framework standards. Clinical observation of patients lasted for six months following the procedure. Before the procedure of autotransplantation as well as every two weeks during the observation period, the assessment of the size of ulcers was carried out using digital photography and a planimetric method using the Visitrack device. During the entire study, patients received standard local and general treatment, including oral phlebotropics, compression therapy and saline solution.

Cell Phenotype Analysis

After every tissue processing procedure, 0.5 mL of the cell suspension concentrate remaining from the enzymatic preparation was intended for cell phenotype analysis. The phenotype of cells separated from adipose tissue was evaluated with the use of phycoerythrin-conjugated mouse monoclonal antibodies: CD73, CD90, CD105, CD34, CD146, CD31 and CD45 (BD Biosciences, USA). After labeling, according to manufacture' instructions and careful washing, cells were analyzed by flow cytometry using the FacsCalibur instrument (Becton Dickinson, CA, USA). Percentage of positive cells was evaluated with CellQuest software (Becton Dickinson, CA, USA).

Statistical Analysis

Statistical analysis was performed using GraphPad Prism software (GraphPad Software La Jolla, CA, USA). The relationships between different variables of interest were assessed by Pearson correlation. Statistical significance was set at $p < 0.05$.

Results

Phenotype of Isolated Cells

Flow cytometry was applied to evaluate cell phenotype separated from the adipose tissue for autotransplantations of patients with VLUs. Figure 1 shows the phenotype of separated cells and average percentage of cells of interest. The percentage of cells expressing the MSC markers $CD73^+$, $CD90^+$ and $CD34^+$ cells was 65–82% in the samples. About 20% $CD31^+$ and $CD45^+$ cells were found in samples.

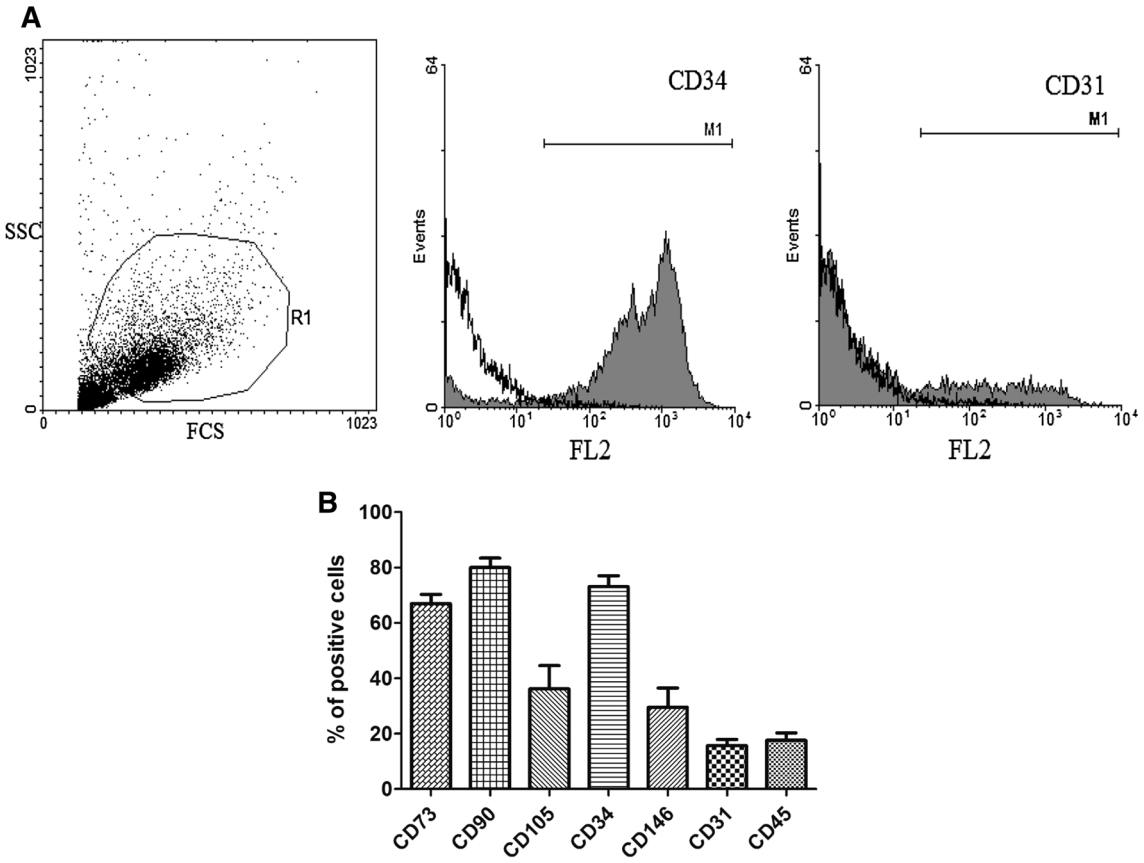

Fig. 1 Phenotype of separated cells and average percentage of cells. Phenotype and average percentage of cells separated from patient's adipose tissue by automated cell separator CELLUTION 800 (Cytori Therapeutics, USA) as evaluated by CellQuest software (Becton Dickinson, CA) and presented by WINMDI 2.8 program. (**a**) Undamaged cells were gated on the base of FSC and SSC parameter (R1). Percentage of labeled cells was evaluated using marker (M1) for two example antibodies CD34 and CD31, to discriminate labeled cells from control cells (labeled with isotype control). (**b**) Average percentage of positive cells isolated from 11 patients

Clinical Observation

Data describing the size of the wound at the time of inclusion in the study, the number of cells given to individual patients during the autotransplantation procedure and the observed effect of treatment in the form of percentage change of the wound area were all collected and are presented in Table 2. The preparation obtained from the separator contained an average of $5.6\times10^6\pm4\times10^6$ cells per milliliter of cell suspension concentrate. Patients were given from 8.0×10^5 to 2.3×10^7 cell suspension concentrate, among which there was 45–92% of cells with a stem cell phenotype expressing the $CD34^+$ antigen.

After therapy, an improvement in the clinical condition was observed in 75% of ulcers. Complete healing occurred in 25.0% of ulcers ranging 10.0–25.5 cm^2 (mean: 19.8 cm^2). A completely healed wound is shown as an example in Fig. 2a. In 50.0% of ulcers ranging 17.8–66.7 cm^2 (mean: 38.3 cm^2), a reduction in wound area greater than 50% was observed. In 25.0% of ulcers ranging 85.0–147.5 cm^2 (mean: 119.5 cm^2), there was no change in the size of the wound after autotransplantation (an example is shown in Fig. 2b).

Next, the relationships between different variables of interest were assessed using Pearson correlation analysis and are presented in Fig. 3. Special attention was drawn on a highly significant negative correlation ($p<0.0001$) between wound size and wound closure degree (Fig. 3a). However, no relationship was found between ulcer healing and the total number of cells given in the suspension, the number of cells in the transplanted material per cm^2 or the age of the patient. There was no correlation between the progress of wound healing and sex, body mass index values, the levels of cholesterol, triglycerides and glucose in the serum of subjects (data not shown).

There were no serious side effects during procedure or after the autotransplantation of stem cells in patients. In one patient only a small swelling at the injection site was reported, however, without significant clinical importance.

Table 2 Characteristics of the subjects including wound size at the day of study inclusion and the transplant with the effectiveness of therapy measured by percentage of wound closure

No.	Sex	Age	Wound size (cm^2)	No. of transplanted cells	No. of transplanted cells (cm^2)	$CD34^+$ cells (%)	Wound closure (%)
1	F	74	25.0	8.0×10^5	0.32×10^5	ND	100
2	M	62	34 .0	6.3×10^6	1.85×10^5	ND	> 50
3	F	68	31.0	1.1×10^7	3.55×10^5	70	> 75
4	M	85	34.5	1.4×10^7	4.06×10^5	45	> 50
5	M	56	66.7	5.1×10^6	0.76×10^5	80	> 50
6	F	74	126.5	7.3×10^6	0.58×10^5	92	0
7	F	57	85.0	2.3×10^7	2.71×10^5	65	0
8	F	69	17.8	1.3×10^7	7.3×10^5	70	> 75
9	M	82	147.0	3.0×10^5	0.02×10^5	80	0
10	M	64	24.5	1.2×10^6	0.49×10^5	80	100
11	F	66	46.0	1.0×10^6	0.22×10^5	80	> 50
12	F	66	10.0	1.0×10^6	1×10^5	80	100

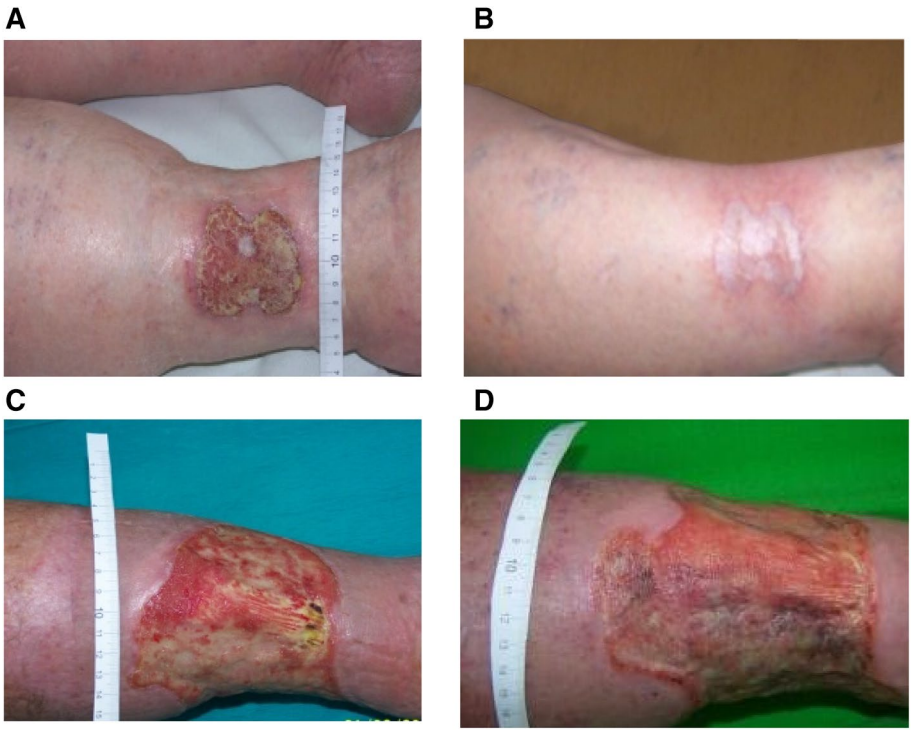

Fig. 2 Representation of the venous leg ulcers wound treatment effects according to wound size area. Documentation showing images of wounds of patients with venous leg ulcers before (**a** and **c**) and 6 months after (**b** and **d**) transplantation of autologous adipose tissue-derived mesenchymal stem cells. Pictures **a**, **b** represent the effect of the therapy of the smallest wound size (10.0–25.5 cm^2). Pictures **c** and **d** represent the effect of the therapy of the largest wound size (85.0–147.5 cm^2)

Discussion

The main goal of regenerative medicine is to restore the anatomical and physiological functions of degenerating tissues by providing elements necessary to repair the body, which can stimulate and support the internal ability of our body to self-regenerate and heal. Preclinical tests and several experimental clinical trials have shown a positive effect of AT-MSC on the healing of chronic wounds (Otero-Viñas and Falanga 2016).

At the same time, the cellular material was prescreened for cellular composition. Criteria of the International Society of Cell Therapy were used as a basis for the assessment; however, the epitopes used in this study for MSC identification are not the only defined (Bourin et al. 2013; Mendicino et al. 2014). MSC are characterized by the expression of surface markers $CD105^+$, $CD73^+$ and $CD90^+$, but also a high percentage of $CD34^+$ cells have been observed, a marker found in MSC freshly isolated from the adipose tissue (Braun et al. 2013). In the cellular material, about 20% of cells with $CD31^+$ phenotype (endothelial cells or endothelial

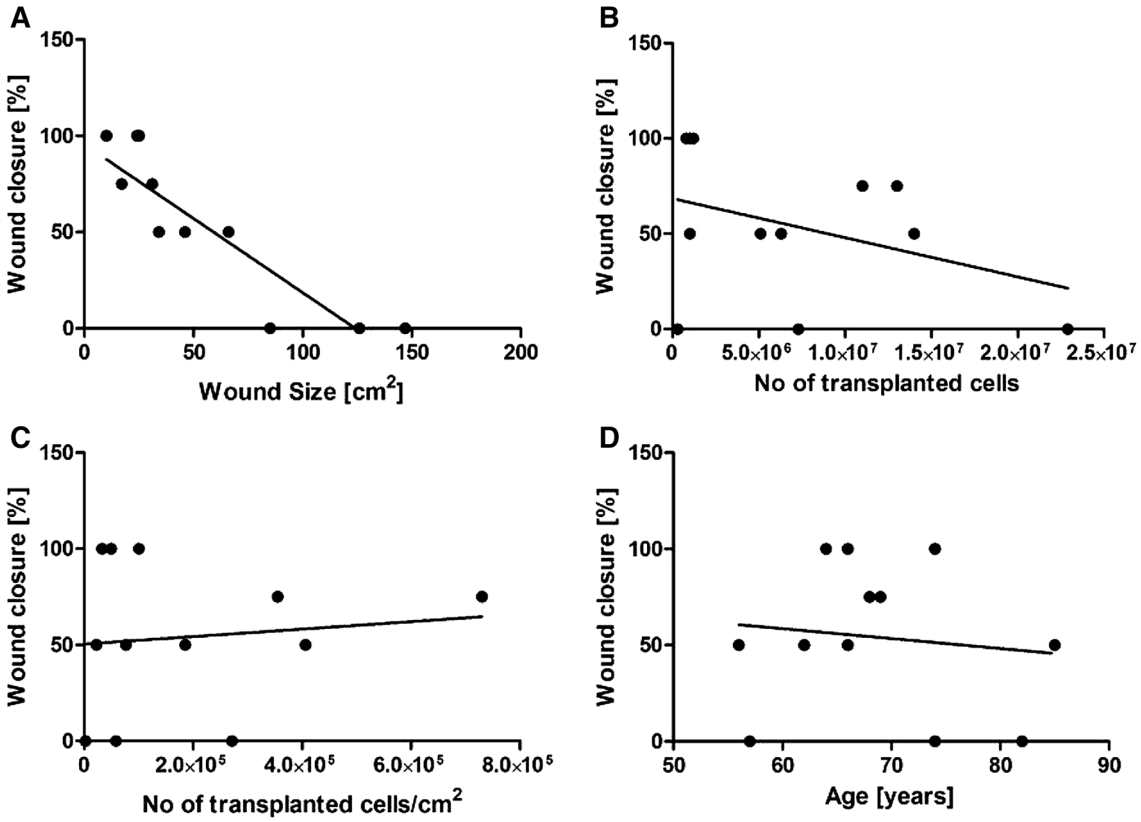

Fig. 3 Relationship of selected parameters to wound closure efficacy. **a** Pearson correlation analysis showing a significant negative correlation between the wound size and the wound closure area ($r = -0.8877$, $p < 0.0001$, $n = 12$). **b** Pearson correlation analysis showing no significant correlation between the number of transplanted cells and the wound closure area ($r = -0.3797$, $n = 12$). **c** Pearson correlation analysis showing no significant correlation between number of transplanted cells injected by cm^2 of wound and the wound closure area ($r = 0.00904$, $n = 12$). **d** Pearson correlation analysis showing no significant correlation between age of the patients and the wound closure area ($r = -0.1210$, $n = 12$)

progenitors) and $CD45^+$ (cells of myeloid origin) were also found. In different works describing the cells isolated from adipose tissue, apart from the MSC, additional populations of blood-derived $CD45^+$ cells, endothelial progenitors and pericytes are usually found (Baer 2014; Yoshimura et al. 2006). Populations of these blood-derived cells usually disappear following expansion of AT-MSC (Baer 2014).

Numerous observations have shown that the efficacy of the applied therapy is influenced by the site of adipose tissue collection from the patient, technology used to obtain MSCs, method of cellular material processing and the area where the cells are delivered to the wound (Camilleri et al. 2016; Ho et al. 2008). In addition, the currently available methods for obtaining AT-MSC are different: from minimal processing by administration of the whole lipoaspirate, to SVF isolation, to ex vivo expansion of the processed lipoaspirate (Zuk et al. 2001). Considering these differences, it is difficult to compare results obtained from various studies.

In our study, a validated device, Cellution 800 from Cytori, with the status of medical equipment, intended for the separation of AT-MSC had been used. Tissue processing took place in a closed system, using a sterile disposable set and the processing enzyme Celase, a legally protected reagent with proteolytic properties optimized for the isolation of cells from the lipoaspirate. Celase reagent is validated to be free of bovine spongiform encephalitis (Fraser et al. 2014). The use of an automated device operating in a closed system allows maintenance of fully defined and repeatable MSC fraction separation conditions from the collected tissue material, ensuring standardization of the method while minimizing the risk of cell contamination at the processing stage. However, some differences in the amount of cells in the suspension concentrate resulting from the separating procedure were seen. To use all the regenerative potential of the cells separated from the patients' adipose tissue, a standard volume of 4.5 mL of the cell suspension concentrate was taken for the autotransplantation procedure, regardless of individual differences.

In our pilot study, patient wounds were injected locally with 3.0×10^5 to 2.3×10^7 autologous AT-MSC and were observed for six months. All patients participating in this experimental study were previously treated using standard

procedures, but without significant clinical improvement or even relapsing. Among the selected relationships between different variables of interest describing the patient and the wound closure, the most interesting one that was highly statistically significant (with $p < 0.0001$) was the correlation between the wound closure rate and the surface wound area. As expected, it was clearly demonstrated that the smaller the wound, the better the results of a single MSC transplantation. According to our knowledge, this is the first known report showing that the positive effect of the use of autologous stromal cells obtained from human body fat in the treatment of VLUs depends largely on the size of the wound. A positive effect of the use of human MSCs isolated from adipose tissue was previously observed to accelerate wound healing in a mouse model of diabetic foot (Amos et al. 2010). There were also a few clinical trials using adipose-derived human stroma cells. In the study of Zollino et al. (2019). centrifuged adipose tissue (CAD) containing progenitor cells was used to treat non-healing VLUs. In experimental arm treated with CAD, advanced dressing and compression the authors showed faster healing time comparing to control arm treated without CAD.

The lack of correlation with number of transplanted cells and number of $CD34^+$ cells in the transplant deserves further discussion. We expected a correlation between wound closure and number of transplanted cells, similar to the studies on the porcine wound model where the therapeutic effect of AT-MSC was demonstrated in a dose-dependent manner (James et al. 2018). In controlled clinical trial using CAD to treat non-healing VLUs, there was also a strong reverse correlation between percent of $CD34^+/CD45^-$ non-hematopoietic cells, respectively, with the healing time and pain (Zollino et al. 2019). The reason for such result may be variation of wound size in the subjects included in the study. Meanwhile, the presented analysis indicate that a positive therapeutic effect depends more on the size of the wound than on the number of cells administered. The question is how many cells should actually be administered to the patient to obtain a positive effect. So far, such studies have not been carried out and determination or methodology for estimating the amount of cells administered still needs to be properly defined. Moreover, in our study, cell suspension cocktail was not standardized to obtain the same amount of cells injected to every patient, because of differences in the wound size. We supposed that, given the same amount of the cell cocktail to different wound areas would not be effective.

Surprisingly, the relationship between wound closure, patient age and the number of transplanted cells per cm^2 was also not found. In elderly patients, reduction of MSC proliferation rate and limitation of their ability to differentiate in vitro were observed (Marędziak et al. 2016; Wagner et al. 2008); whereas in our study, attention is drawn to the lack of relationship between treatment effect and age. Therefore, it does not exclude elderly patients from this treatment, and at the same time suggests that MSC still show biological activity with therapeutic potential in patients with the disease.

In this experiment, the safety of the procedures used was assessed. During procedure and after the use of stromal cells autotransplantation, no serious side effects were demonstrated. Only one patient had a mild edema at the injection site, but with no clinical significance. In a meta-analysis of 5578 patients with chronic lower limb ischemic wounds who received therapies using stromal cells isolated from their own adipose tissue, side effects were demonstrated with an average frequency of 3.3%, including less severe events (such as nodularity/induration, superficial infection and pain) occurring with average frequency of 2.77%, and events of significant clinical significance (including, among others, deep infection and sepsis) with an average frequency of 0.55%, as shown by Zollino et al. (2017). In a phase II randomized clinical trial for the treatment of non-healing VLUs with CAD containing progenitor cells, there were also no major adverse events recorded (Zollino et al. 2019).

The results of our research indicate that the use of autologous MSC is safe and could be an alternative form of therapy for patients with chronic wounds. It has so far been demonstrated in clinical trials that AT-MSC transplantation is a safe and potentially effective method of treatment in Crohn's disease-related skin fistula (Garcia-Olmo et al. 2005, 2009). However, the current lack of uniform standards in the collection, processing and administration of the obtained cellular material makes it difficult to compare existing data regarding the safety and efficacy of the applied AT-MSC-based therapies. Currently, the most frequently used alternative to biological treatment using MSC cells are MSCs of bone marrow origin. The observation is reliable, however, that stromal cells isolated from adipose tissue have significant potential for regeneration of the patient's own tissues. Confirmation, however, is required for the correlation of treatment effects and the size of the wound, as confirmed by this study on a small group of patients. Unexplained, however, from a practical point of view, is the problem of how many cells should actually be administered to the patient to achieve a visible therapeutic effect. It can be assumed that the patient's own MSCs do not stay in the body for a long time, but only initiate the process of wound healing by releasing numerous biologically active factors in the local environment. The improved closure of smaller size wounds observed in our study may be based on the paracrine effect caused by the MSCs. In vitro studies have revealed the influence of factors secreted by the AT-MSC on the increase of activation, proliferation and collagen synthesis as well as migration ability of human dermal fibroblasts and keratinocytes (Kim et al. 2007; Lee et al. 2012; Walter et al. 2010). However, MSC secretome appears to vary significantly, depending on many factors, such as the age of the host (Praveen Kumar et al.

2019). Moreover, for the observed effect, also different cells, not only the $CD34^+$ cells, may be responsible. However, this still needs to be defined.

We want to emphasize that our study was important in demonstrating the efficacy and safety of the therapy. It seems that this kind of treatment and evaluation of the effects is relatively simple, yet at the same time a precise and objective model for this type of research. Our pilot study presents the largest group of patients with VLUs treated with MSCs autotransplantation in Poland. However, more studies with randomized, control group are needed. We would like to point out that there were no adverse reactions to the autologous implantation of MSCs.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki declaration and its later amendments or comparable ethical standards.

Informed consent Informed consent was obtained from all individual participants included in the study.

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