Am J Transl Res 2021;13(8):9495-9504
www.ajtr.org /ISSN:1943-8141/AJTR0134350

Original Article

A pilot study using cell-mixed sheets of autologous fibroblast cells and peripheral blood mononuclear cells to treat refractory cutaneous ulcers

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Received April 16, 2021; Accepted June 24, 2021; Epub August 15, 2021; Published August 30, 2021

Abstract: Background/Aims: We invented a cell-mixed sheet consisting of autologous fibroblast cells and peripheral blood mononuclear cells (PBMNCs) to treat refractory cutaneous ulcers. These sheets secrete the growth factors needed throughout the wound healing process in animal models. Methods: We performed this study as a pilot phase I clinical trial (UMIN-CTR: UMIN000031645). Fibroblast cells were isolated and cultured from the oral tissue, and PBMNCs were collected by apheresis. A cell-mixed sheet was prepared by co-culturing these collected cells for 3 days. The primary observation index was safety, including all adverse events. Additional observation indices were wound healing over 1, 3, and 6 months; wound healing rate at 7 days and 1, 3, and 6 months. Results: Six patients with venous leg ulcers (VLUs) were enrolled in the study, including three patients who were treated with the cell-mixed sheet transplantation. One patient was excluded because no fibroblast cells grew from the oral tissue culture, and other two were excluded because the growth factor secreted from mixed-cell sheets did not reach the reference value. The VLUs of two patients who received the cell-mixed sheet transplantation healed, and the VLU in one patient decreased in size. Conclusions: This pilot study demonstrated that cell-mixed sheets might be a new topical intervention to treat VLUs. However, it was also suggested that this treatment might be limited when using autologous cells collected from patients with VLUs. Therefore, it may be necessary to use high-quality allogeneic cells instead of autologous cells to improve the feasibility of this treatment.

Keywords: Venous leg ulcer, cell sheet, fibroblast cell, peripheral blood mononuclear cell

Introduction

Refractory skin ulcers are caused by several factors such as pressure and congestive disorders (including venous insufficiency), diabetes mellitus, and peripheral arterial disease; therefore, treatments for refractory skin ulcers are determined based on their causative factor [1-4]. For chronic wounds for which conventional therapy is ineffective, advanced therapies such as extracellular matrices, growth factors, negative pressure wound therapy and engrafted skin are often used. However, selecting these advanced therapies is not very often evidence-based, and the development of more effective treatments is necessary [5, 6]. We invented a cell-mixed sheet consisting of autologous fibroblast cells and peripheral blood mononuclear cells (PBMNCs) to treat refractory cutaneous ulcers. These sheets secrete necessary growth factors throughout the wound healing process and were found to be effective for ulcers in animal models [7-9]. Therefore, this cell sheet required a clinical pilot study.

Fibroblast cells were isolated and cultured from the oral tissue, and PBMNCs were collected by apheresis. A cell-mixed sheet was prepared by

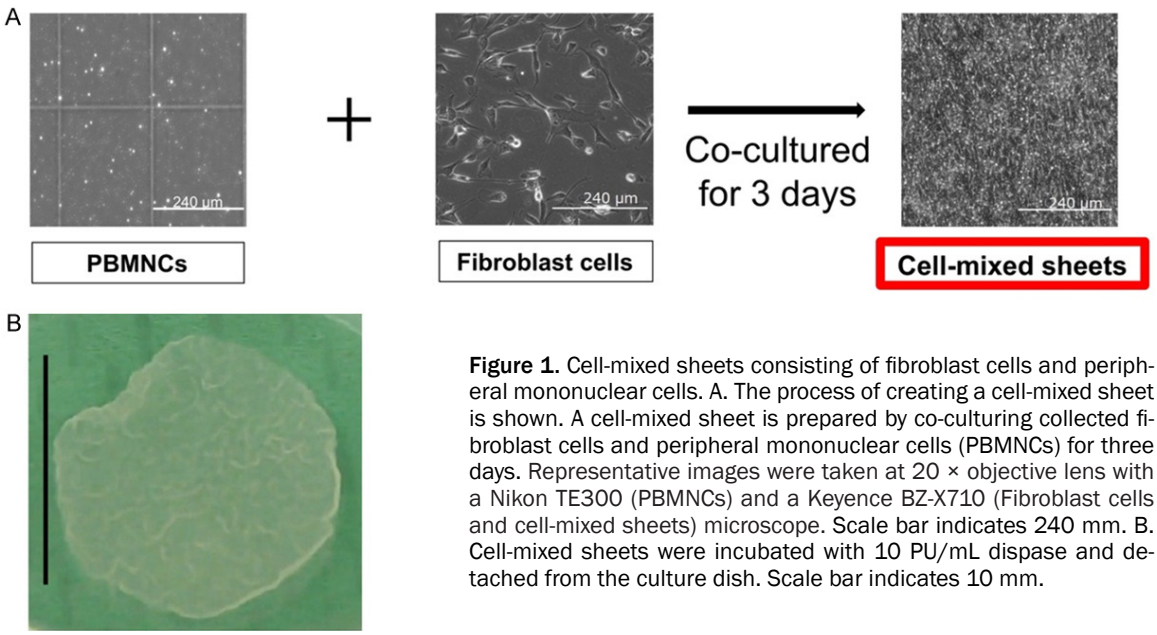

Figure 1. Cell-mixed sheets consisting of fibroblast cells and peripheral mononuclear cells. A. The process of creating a cell-mixed sheet is shown. A cell-mixed sheet is prepared by co-culturing collected fibroblast cells and peripheral mononuclear cells (PBMNCs) for three days. Representative images were taken at 20 × objective lens with a Nikon TE300 (PBMNCs) and a Keyence BZ-X710 (Fibroblast cells and cell-mixed sheets) microscope. Scale bar indicates 240 mm. B. Cell-mixed sheets were incubated with 10 PU/mL dispase and detached from the culture dish. Scale bar indicates 10 mm.

co-culturing these collected cell species for three days (**Figure 1**). These sheets were then transplanted to venous leg ulcers (VLUs). In our department, VLUs have been treated with compression therapy for several years; therefore, we had sufficient data to compare the results from cell-mixed sheets [10]. Furthermore, the transplantation of the cell sheets to the medial side of the lower leg, which is a common site for VLUs, is a simple procedure. The ability of cell-mixed sheets to heal VLUs was evaluated in this single-arm pilot study. We hypothesized that cell-mixed sheet therapy could effectively heal wounds via the paracrine effects on the wound bed.

Materials and methods

Study design

This pilot study as a clinical trial was performed from March 9, 2018, to February 4, 2021, at a single hospital. The objective of this study was to evaluate the ability of cell-mixed sheets for treating six patients with VLUs in an outpatient setting. Duplex ultrasonography was performed in all patients. All patients were found to have superficial or deep venous reflux at baseline. This study included patients with VLUs refractory to surgery (catheter ablation or stripping). The primary observation index was the safety of the cell-mixed sheets, including the presence or absence, frequency, and severity of adverse events. Additional observation indices were wound healing at 7 days and 1, 3, and 6 months, i.e., wound healing rate and patient-reported pain at 7 days and 1, 3, and 6 months based on the visual analog scale. No sample size calculation was used in this study, but the study included six adult patients with a VLU. This study was approved by the Certified Committee for Regenerative Medicine of Yamaguchi University Hospital and the Ministry of Health, Labour and Welfare (YS2017-001; jRCTb0601-90034). The informed consent was obtained from all study participants and the study was conducted in accordance with the Declaration of Helsinki and the Act on the Safety of Regenerative Medicine. This study was registered at the UMIN Clinical Trials Registry (UMIN-CTR: UMIN000031645). The inclusion and exclusion criteria are shown in **Table 1**. Patients of at least 20 years of age with a VLU on the leg were included in this study. Each VLU was present for at least six months before the cell-mixed sheet treatment. The exclusion criteria were designed to exclude patients with critical limb ischemia (CLI).

Cell-mixed sheet application procedures and follow-up

All procedures were performed at our hospital. Approximately 200 mL of whole blood was drawn from each patient to obtain 100 mL of autologous serum. A dentist excised and col-

Table 1. Inclusion and exclusion criteria in this study

Inclusion criteria	Exclusion criteria
1. Patients aged between 20 to 85 years at the time of obtaining consent. 2. Patients with venous insufficiency according to the following criteria: 1) Patients with VFI of 2.0 mL/s or more, EF of 40% or less, or RVF of 35% or more in APG examination. 2) Patients with regurgitation in superficial or deep veins by lower limb vein echography (duplex method). 3. Patients who were difficult to treat with surgery (such as stripping), external medicine, or compression therapy. 4. Patients with a single wound and a skin ulcer with a major axis of 5 cm or less, or patients with skin ulcers whose wound area did not exceed 23.04 cm^2 (the area of eight cell-mixed sheets).	1. Patients suffering from critical limb ischemia. 2. Patients suffering from malignant neoplasm. 3. Patients suffering from bacterial infections such as treponema palladium, chlamydia, gonorrhea, or tuberculosis. 4. Patients suffering from infections such as hepatitis B or C, human immunodeficiency virus, human T lymphotropic virus type 1, and parvovirus B19. 5. Patients with allergies to local anesthesia or aminoglycoside antibiotics. 6. Patient with hemoglobin: < 8.0 g/dL, Platelet: < 50,000/μL, Prothrombin time: < 40%. 7. Patients with or suspected of having sepsis. 8. Patients with congenital coagulopathy, antiplatelet drug use, or anticoagulant use. 9. Patients who have experienced other cell or gene therapy. 10. Patients participating in other clinical trials. 11. Patients undergoing hemodialysis. 12. Patients who developed unstable angina, myocardial ischemia, or cerebral infarction within 3 months. 13. Pregnant or nursing women. 14. Patients with allergy of aminoglycoside antibiotics. 15. Patients determined to be ineligible for this clinical study by research attending physician.

Abbreviations: VFI = venous filling index; EF = ejection fraction; RVF = residual volume fraction; APG = air plethysmography.

lected a 5 mm × 3 mm × 2 mm (major axis × minor axis × depth) oral tissue sample from each patient under local anesthesia. In the Regeneration and Cell Processing Center (CPC) manufacturing department, the oral tissue was shredded, and approximately 1 mm was attached to a cell culture dish with a culture solution containing collagenase (5% autologous serum). The cells were cultured for 10 to 36 hours before the medium was changed. The cells were cultured for an additional 10 days to allow for sufficient fibroblast growth. The autologous fibroblasts were cultured for approximately three weeks until the number of cells required for cell sheet preparation was obtained. Autologous PBMNCs were collected three days before transplanting the cell-mixed sheets. Approximately 2,000-3,000 mL of peripheral blood obtained from each patient was separated for 60-90 minutes using a Cobe Spectra cell separator (Terumo BCT Inc., Lakewood, CO, USA). At least 3.3×10^7 PBMNCs were collected from approximately 100 mL of the peripheral blood. A 5.0×10^5/mL (2.0×10^6/4 mL) fibroblast suspension was prepared in a serum-free medium using self-cultured fibroblasts. PBMNCs were prepared as a 6.7×10^5 cells/mL (2.0×10^6 cells/3 mL) suspension using serum-free medium. The autologous serum was diluted by adding 1.6 mL autologous serum to 18.4 mL of serum-free medium. For the preparation of one cultured human autologous cell-mixed sheet, 4 mL of fibroblast suspension, 3 mL of PBMNC suspension, and 1 mL of diluted autologous serum were placed in each well of a 6-well plate. The final concentration of autologous serum was 1%. The mixture was incubated at 37°C in 5% CO_2 for two days and at 33°C, 5% CO_2, and 2% O_2 for one day for the treatment of hypoxic preconditioning, which has been reported previously by us [11, 12]. Using the cultured cells and autologous serum, up to 12 cultured human autologous cell-mixed sheets could be prepared for transplantation. The CPC quality department performed a cell surface antigen analysis and measured the vascular endothelial growth factor (VEGF) concentration in the supernatant of the cultured human autologous cell-mixed sheets on the first and third day of culture. Cultured human autologous cell-mixed sheets that were determined ready for transplantation were treated with 10 PU/mL dispase before transplantation. Cultured human autologous cell-mixed sheets were transplanted to the wound using a pipette. The wound was protected with a covering material depending on the wound's condition, and the limb was compressed with an elastic bandage. During each visit, the maximal perpendicular diameters of the VLU were measured, and these values were multiplied to determine the area of the ulcer, as previously described [10]. The VLU was considered healed once epithelialization of the entire wound surface was achieved.

Statistical analyses were performed using Stata/IC software, version 15.1 (StataCorp, College Station, TX, USA). Descriptive statistics were used to analyze the patient and wound characteristics and the pain data. The percent of wound area reduction was calculated at 7 days and 1, 3, and 6 months.

Results

Six patients were enrolled in the study. **Table 2** shows the patient and wound characteristics. The mean VLU duration was 17 ± 11 months (range: 6-32 months). The mean baseline wound area was 13.3 ± 5 cm^2 (range: 7.4-21.6 cm^2).

Oral tissue was harvested from all six patients. However, fibroblast growth was not detected in one patient and was therefore excluded from the study. The remaining five patients underwent PBMNC collection. VEGF secretion from the cell-mixed sheets did not attain the reference value in another two patients who were excluded from the study. Therefore, only three patients received cell-mixed sheet transplants. The changes in wound condition, wound healing rate, and pain scores for each patient are shown in **Figures 2**-**4**.

Patient number 1 had a 7.4 cm^2 VLU that had been open for 9 months and had not responded well to compression therapy. However, the VLU closed one month after the cell-mixed sheet transplantation (**Figure 2**).

Patient number 2 had two VLUs with cutaneous necrosis covering a total area of 14.8 cm^2. The VLUs had been open for 6 months and had not responded well to compression therapy. We performed debridement and transplanted the cell-mixed sheets. The VLU initially increased in size. However, the granulation eventually

Table 2. Patient and wound characteristics

Patient No.	Age, y	Sex	BMI	Comorbidities and problems	Duration of ulcer, months	Reflux vein	Ulcer location	Initial wound area, cm^2	Cell-mixed sheets transplantation
1	66	F	20	Hypertension Long standing time	9	Popliteal vein	Left median ankle	7.4	Possible
2	41	M	39	Hypertension Long standing time	6	GSV before stripping	Left shin	14.8	Possible
3	74	F	25	Osteoarthritis of bilateral knees	24	GSV before ablation	Left median ankle	16.4	Possible
4	83	F	22	Hypertension Dyslipidemia, Rheumatoid arthritis Osteoarthritis of bilateral hips and knees	32	GSV before ablation	Left posterior calf	11.1	Impossible (no fibroblast cells growth)
5	63	M	26	Hypertension Diabetes mellitus Dyslipidemia Long standing time	10	GSV before ablation	Left dorsum of the foot	8.6	Impossible (poor VEGF secretion)
6	60	M	28	Diabetes mellitus Dyslipidemia Chronic hepatitis Long standing time	30	GSV before ablation	Left calf	21.6	Impossible (poor VEGF secretion)

Abbreviations: BMI = body mass index; F = female; M = male; GSV = great saphenous vein.

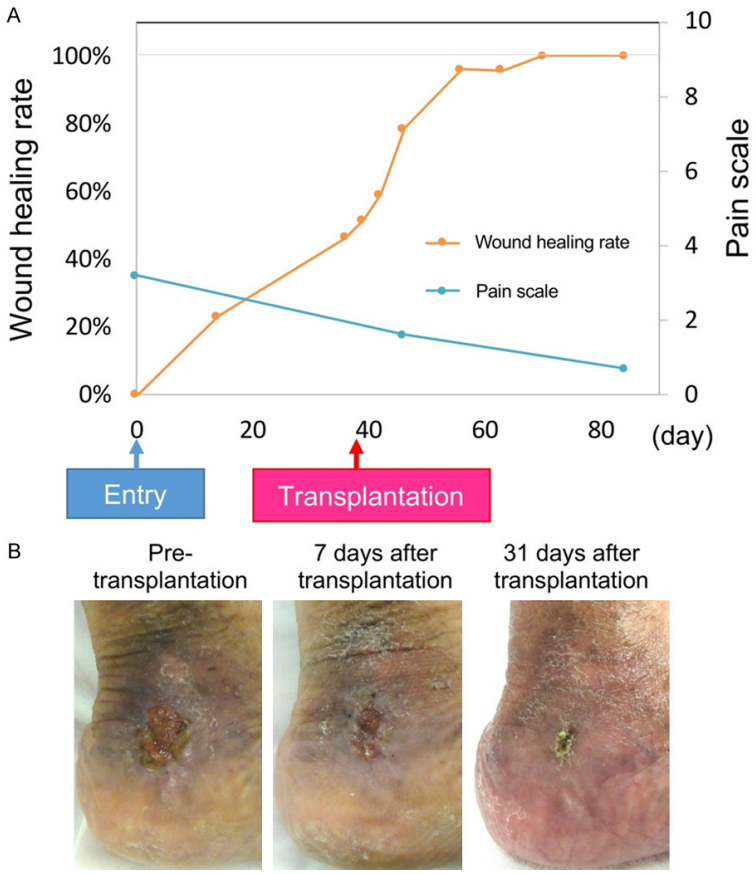

Figure 2. Wound closure in patient number 1. A. The time course of wound healing and reported pain scores is shown. B. Representative images of the healing of the VLU treated are shown. The VLU achieved closure 31 days after the cell-mixed sheet transplantation.

improved. At 1 month, a third VLU appeared at a different site, and the subject reported severe pain at the site of the new VLU. Despite the risk of therapy bias, the patient received hyperbaric oxygen therapy (HBOT) for 3 months. The VLUs that were transplanted with cell-mixed sheets closed after 5 months (**Figure 3**).

Patient number 3 had a deep 16.4 cm^2 VLU that had been open for 2 years and had not responded well to compression therapy or catheter ablation of the great saphenous vein. Although the wound did not close within six months of the cell-mixed sheet transplantation, it decreased in size to 3.0 cm^2 (**Figure 4**).

All three patients reported decreased pain compared to baseline. All the wounds had good granulation growth. In particular, number 1 and 3 showed a rapid reduction in total wound area after transplantation (**Figures 2** and **4**). No adverse events occurred in any patient.

Discussion

This study assessed the efficacy of a single, outpatient application of cell-mixed sheet treatment for VLUs. VLUs are typical chronic wounds, occurring in > 2% of the general population [13-15]. Compression therapy has been considered the gold standard for VLU treatment for centuries [16]. Advanced wound care therapies by engineered skins are commercially available to treat VLUs in Western countries [17-19]. These skin grafts provide healthy tissue that can incorporate and facilitate closure of the wound bed [20]. However, the metabolic demands of the transferred tissue during the early engrafting period may not be met by the chronic wound bed, which is attributed to the failure rate of these grafts [21-25]. Therefore, there is currently no treatment that significantly improves refractory VLUs that are unresponsive to compression therapy.

Cell delivery strategies, namely cell-based therapies, enable the stable supply of growth factors and cytokines for angiogenesis in ischemic tissues [26, 27]. Previously, we performed the first human trial involving transplantation of bone marrow mononuclear cells (BMMNCs) in patients with CLI. Our study highlighted the feasibility of cell-based therapeutic angiogenesis in patients with CLI, even though the number of patients in our previous study was low [28]. Following our study, several clinical trials have been conducted using bone marrow-derived cells in patients with CLI. In recent years, PBMNCs have also been used for cell-based therapeutic angiogenesis in patients with CLI. PBMNCs can be isolated from patients more easily and safely than BMMNCs while displaying similar therapeutic efficacy [29]. To improve the survival rate of transplanted cells in ischemic tissue, we used a previously reported method of hypoxic preconditioning to promote neovascularization [11, 12]. PBMNCs were used instead of bone

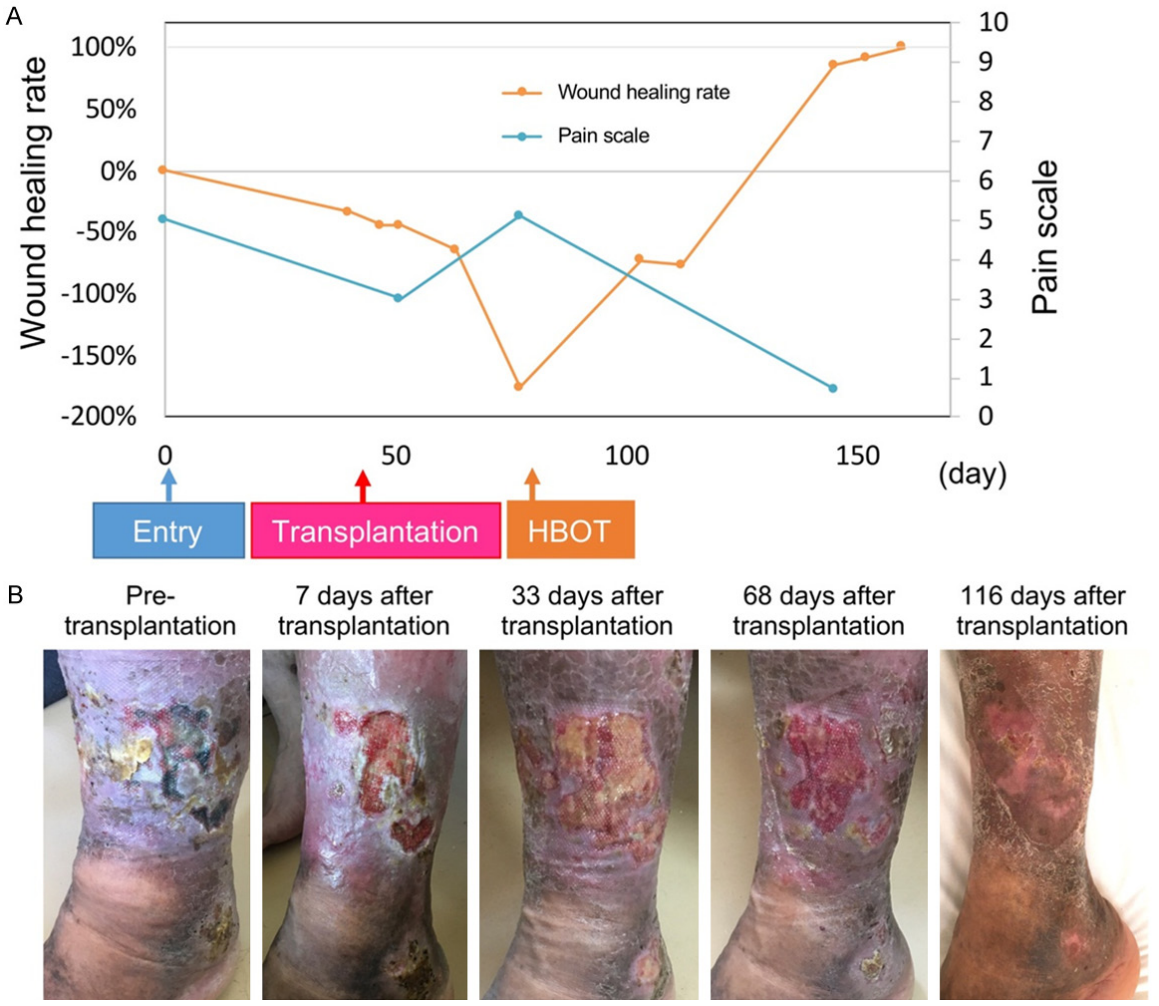

Figure 3. Wound closure in patient number 2. A. The time course of wound healing and reported pain scores is shown. B. Representative images of the healing of the VLU treated are shown. The VLU achieved closure at 116 days after the cell-mixed sheet transplantation.

marrow cells as collecting PBMNCs is less invasive. Our previous study examined the therapeutic effects of preconditioned PBMNCs. Hypoxically-pretreated PBMNCs improved microvessel density and limb blood flow in hindlimb ischemia models [30, 31]. Cell sheet technology has been developed to improve the engraftment of transplanted cells in grafted regions [32]. A primary factor responsible for the insufficient repair of refractory ulcers is the poor retention of grafted cells or artificial skin in wounded areas. Therefore, we used cell sheet technology to treat refractory cutaneous ulcers. Cell-mixed sheets consisting of autologous fibroblast cells and PBMNCs were formed. These sheets were prepared under hypoxic conditions. They provided the growth factors needed throughout the wound healing process and effectively treated ulcers in animal models [7-9].

In this study, three patients with VLUs were treated with cell-mixed sheet transplantation. Three patients were excluded due to no fibroblast growth in the oral tissue specimen (n = 1) or poor growth factor in the cell-mixed sheets (n = 2). The failure to obtain fibroblasts and promote growth factor secretion during the formation of the cell-mixed sheets is a challenge that must be overcome before the widespread use of this therapy. We plan to examine the reason for this failure using the preserved sample. Thus far, we believe that the patients' poor overall health and healing ability may have contributed to these limitations, suggesting that

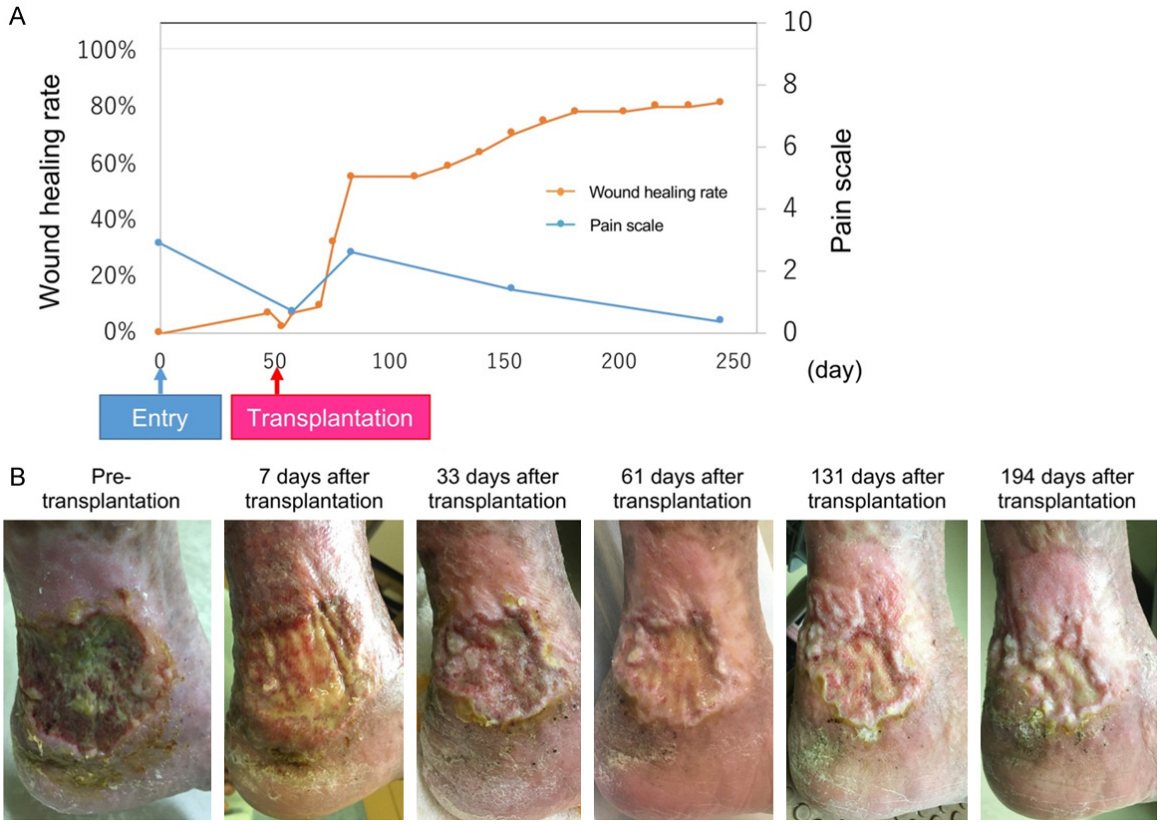

Figure 4. Wound closure in patient number 3. A. The time course of wound healing and reported pain scores is shown. B. Representative images of the healing of the VLU treated are shown. The VLU did not heal completely after 6 months of cell-mixed sheet transplantation; however, the VLU decreased in size.

allogenic transplantations may be necessary. Our previous study suggested that the efficacy of allogenic cell transplantation is similar to that of autologous cell transplantation [33].

In this study, the VLUs healed in two patients who received the cell-mixed sheet transplantation. In the third patient, the VLU decreased in size. Patient number 2 required adjuvant HBOT. The granulation of the patient's VLU improved between the cell-mixed sheet transplantation and the initiation of HBOT; however, the VLU area increased slightly. All three patients reported improvement of pain during the healing process, and no serious adverse events were reported. These findings suggest that cell-mixed sheets are a safe and viable mode of treatment for chronic VLUs.

Although our study reports what could be a revolutionary treatment method for VLUs, the results should be evaluated in the light of a few limitations. First, this study did not compare the cell-mixed sheet transplantation with other products or procedures. Furthermore, the low number of completed procedures in this study limits the power of our originally planned comparison with previously reported data from compression therapy [10]. A randomized controlled trial should be conducted to obtain more information regarding cell-mixed sheet treatment.

Conclusion

This study demonstrated that cell-mixed sheet transplantation might be a safe and somewhat effective treatment for chronic VLUs refractory to standard compression therapy. However, it was also suggested that this treatment might be limited when using autologous cells collected from patients with VLUs. In our case, three of the six patients enrolled had to be excluded because sufficient cell-mixed sheets could not be grown for treatment. Therefore, future studies should consider using high-quality allogeneic cells instead of autologous ones to improve the feasibility of this treatment.

Disclosure of conflict of interest

None.

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