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ARTICLE

Effectiveness of the combination of fat grafting and injection on radiation ulcer healing

Michiko Fukuba[a], Hiroshi Uozaki[b] and Yuzo Komuro[a]

[a]Department of Plastic, Oral and Maxillofacial Surgery, Teikyo University School of Medicine, Tokyo, Japan; [b]Department of Pathology, Teikyo University School of Medicine, Tokyo, Japan

ABSTRACT

Fat injections aid in the healing of radiation-induced skin damage. We hypothesized that the direct application of fat grafts to the surfaces of radiation-induced ulcers is also effective. Here, we aimed to evaluate the effectiveness of a combination treatment comprising fat injections around ulcers and fat grafts on ulcer surfaces. The dorsal skin of inbred rats was irradiated at a single dose of 20 Gy before producing ulcers. After the inguinal fat was harvested using the Coleman technique, the rats were divided into four groups: Group 1, ulcer wounds were covered using dressing materials and staples only; Group 2, fat was injected around the ulcers using a cannula; Group 3, fat was grafted onto ulcer surfaces; and Group 4, a combination of fat injection around the ulcers and fat grafts onto ulcer surfaces was employed. The mean healing time (± standard deviation) of each group was as follows: Group 1, 16.0 ± 2.2 days; Group 2, 14.5 ± 2.0 days; Group 3, 15.2 ± 1.7 days; and Group 4, 13.4 ± 1.0 days. The healing time of Group 4 was significantly shorter than that of Group 1 ($p = .0005$) and Group 3 ($p = .023$). In both groups that received fat grafts, fat tissue was observed in the dermis on hematoxylin-eosin-stained slides at 4 and 8 weeks after the ulcers were created. In conclusion, the combination treatment of fat grafted onto ulcer surfaces and injected around ulcers was effective in accelerating the epithelization of radiation-induced ulcers.

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Radiation; wound; ulcer; fat; adipose; graft

Introduction

Radiation-induced skin damage can be caused by radiotherapy, treatments performed under fluoroscopy and exposure to radioactive material from a nuclear power plant accident. The first stage of radiation-induced skin damage is acute radiodermatitis, which is an inflammatory condition involving erythema, edema, epidermal thickening, pruritus and desquamation. Gradually, the fibrotic process progresses to chronic radiodermatitis, which is characterized by decreased elasticity, hyperpigmentation and ulceration. Radiation-induced ulcers exhibit poor healing and are commonly treated with topical ointments and free flap reconstruction. Because the effective treatment of radiation-induced ulcers remains challenging, alternative therapeutic methods are needed.

The effects of fat grafting on radiation-induced skin damage have been reported by many authors and include the attenuation of radiation-induced fibrosis [1] and the alleviation of skin damage [2,3]. Fat tissue contains adipose-derived stem cells (ASCs), which are reported to secrete multiple cytokines, including angiogenic and antiapoptotic factors [4]. Fat grafts can deliver these substances to damaged tissue to improve skin quality following irradiation. The most commonly used fat-grafting technique is the injection method. While a few studies have examined the topical application of stem cells to chronic ulcers [5], none have investigated the application of purified fat grafts to the surfaces of radiation-induced ulcers. We hypothesized that the application of purified fat grafts directly onto radiation-induced ulcer surfaces might have a curative effect via facilitation of the direct contact between the growth factors released from fat tissue and the surfaces of the ulcers. In most of the previous studies of fat grafting, human fat was used as a graft material in immunocompromised animals; however, these conditions do not match with those found in the clinical setting. In the present study, we used a model involving fat from inbred rats that was designed to reproduce clinical conditions and obtain more clinically relevant results, with a specific aim to investigate whether the application of fat grafts to the surfaces of ulcers would be effective at promoting the epithelization of radiation-induced ulcers.

Materials and methods

Nine-week-old F344/DuCrlCrij inbred rats (Oriental Yeast Co., Ltd., Japan) weighing 170–220 g were used in this study. To rule out any potential variability in skin reaction between the sexes, only male rats were used. All procedures were performed under general anesthesia, which was induced in an airtight ventilated chamber with a mixture of 3% isoflurane and air. Rats were anesthetized with an intraperitoneal injection of a murine anesthetic cocktail consisting of midazolam (2 mg/kg), butorphanol (2.5 mg/kg) and medetomidine (0.15 mg/kg). Local anesthesia was induced with 1% lidocaine for the wound creation, stab incisions and tissue harvesting. The animals were held in single cages under standard environmental conditions and nourished with freely available standard food pellets and water. This study was approved by the Teikyo University Ethical Committee (No: 17–003).

CONTACT Michiko Fukuba michikofukuba@med.teikyo-u.ac.jp Department of Plastic, Oral and Maxillofacial Surgery, Teikyo University School of Medicine, 2-11-1 Kaga Itabashi-ku, Tokyo, Japan
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Experimental groups

The day after the X-ray irradiation procedure, a wound was made in the center of the irradiated area of each rat. The rats were then randomly divided into the following four groups of nine rats each: Group 1, the wounds were covered using dressing materials and staples; Group 2, fat was injected around the ulcers using a cannula; Group 3, fat grafts were applied to the surfaces of the ulcers; and Group 4, a combination of fat injection around the ulcers and grafting onto the surfaces of the ulcers was employed.

During the 8-week study period, three rats in Group 1, two rats in Group 2 and two rats in Group 4 unexpectedly died, probably due to the effects of general anesthesia.

Irradiation and ulcers

We modified a previously described rat radiation-induced ulcer model [6]. For the irradiation procedure, the rat's dorsal skin was shaved using electric clippers before placing the rat face down on a turntable for delivery of a single dose of 20 Gy X-rays to the dorsal skin using an MBR-1520R-3 system (Hitachi Medical Corporation, Tokyo, Japan). The distance between the radiation source and the rat was 25 cm, and the irradiation was performed at 150 kV and 5 mA, in the presence of a filter composed of 0.5 mm aluminum + 0.3 mm copper. The dose rate was 0.9 Gy/minute. The animals were shielded by a 4-mm-thick lead plate containing two holes of 2 cm in diameter (Figure 1(A)). The irradiated areas were marked. The day after the X-ray irradiation, cutaneous full thickness wounds were made in the center of each irradiated region using an 8-mm disposable biopsy punch (Kai Industries, Japan) (Figure 1(B)). The wounds were made approximately equidistantly from the forelimb and hindlimb because a preliminary experiment revealed that the healing speed nearer the forelimb differed from that nearer the hindlimb.

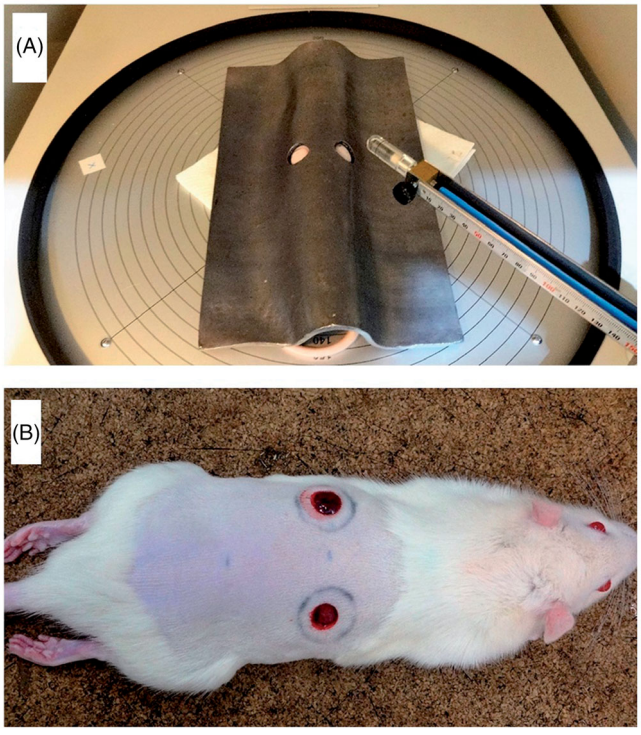

Figure 1. Irradiation and wounding ulceration setup. (A) The 4-mm-thick lead plate containing two holes (diameter: 2 cm) that was used to shield the dorsal area not targeted for irradiation. (B) Representative image showing the positioning of cutaneous full thickness wounds made in the center of the irradiated regions using an 8-mm biopsy punch.

Fat harvesting and grafting

The day after the irradiation procedure, both inguinal areas were shaved, and the inguinal fat was harvested from all of the rats (including the control group) and processed using the Coleman technique [7]. The harvested fat tissue was separated into three layers by centrifugation at 1200 g for 3 min; the middle layer was composed of refined fat tissue, which was partitioned into 1-mL syringes. In groups 2 and 4, three stab incisions were made in the dorsal region, through which the refined fat tissue was injected into the subcutaneous layer around each ulcer target using a blunt cannula. With each pass of the cannula, 0.02 mL refined fat tissue was injected, for a total of 0.08 mL around each ulcer. In groups 3 and 4, 0.02 mL refined fat tissue was grafted onto the surface of the ulcer. All of the ulcers were covered using wound-dressing material (2.5 cm × 2.5 cm; Hydrosite, Smith & Nephew, UK) and staples (Appose, Covidien, USA). A harmless bitter solution (Bitter Apple, Bitter Apple Company, USA) was sprayed onto the dressings to prevent the rats from removing them.

Healing time

We examined the ulcers under general anesthesia at 7, 10, 13, 16, and 19 days after they were created and treated. Photographs of the ulcers were taken during every examination. We defined healing as a combination of ulcer constriction and epithelization.

Histologic examination

A 2 × 2 cm region of skin on the left side of each rat, which included the lesion, was excised 4 weeks after treatment; the skin including lesion on the right side was excised at 8 weeks. The skin samples were fixed in 10% buffered formalin and embedded in paraffin. Sections (3-μm-thickness) were sliced from the paraffin blocks and processed for hematoxylin-eosin and other types of staining.

Epidermal thickness and the number of blood vessels

The central part of each scar was divided using five equally spaced points, and epidermal thickness was measured at each point. The stratum corneum was excluded from these measurements.

Blood vessels were counted in five 200 × 400-μm areas each from the upper and lower regions of the central part of the scar dermis. Immunohistochemical staining of CD34, a marker of endothelial cells, was also carried out to assist with counting the number of blood vessels using an anti-CD34 antibody (EP373Y, Abcam, UK) and Histofine Simple Stain Rat MAX PO (MULTI; Nichirei Biosciences, Japan) as a secondary antibody.

Scar index

Sirius red staining was used to detect collagen structures. The slides were stained in 1% Sirius red solution for 10 min followed by removal of background or excess stain with pure alcohol. Stained sections were examined under the microscope using a polarizing filter. Each section contained the center of a scar. The WinROOF software (Mitani Corp., Japan) was used to quantify colors within the yellow-green and red-orange spectra. As a wound heals, thick type I collagen is replaced by thin type III collagen. Sirius red staining enhances the natural birefringence of collagen bundles; thus, the color of collagen fibers that are stained with Sirius red and viewed with polarized light depends upon the

type(s) of collagen present [8]. A scar index was calculated by dividing the area that fell within the red-orange spectrum by the area that fell within the yellow-green spectrum, with higher numbers representing greater fibrosis [9].

Statistics

Data are presented as the mean ± standard deviation (depicted by error bars). JMP pro 14.1 software (SAS Institute Inc., USA) was used to perform the paired t test or the Tukey–Kramer test. p values $<.05$ were considered statistically significant.

Results

Mean healing time

The mean healing times in Groups 1–4 were 16.0 ± 2.2 days, 14.5 ± 2.0 days, 15.2 ± 1.7 days and 13.4 ± 1.0 days, respectively (Figure 2). Healing occurred significantly faster in Group 4 compared with Group 1 (p=.0005) and Group 3 (p=.023) (Figure 3).

Intradermal fat findings

Fat tissue was observed in the dermis in some specimens (Figure 4(A,B)). In Group 1, fat was found in 0/8 slides at 4 weeks and 0/6 slides at 8 weeks. In Group 2, fat was found in 0/7 slides at 4 weeks and 1/7 slides at 8 weeks. In Group 3, fat was found in 9/9 slides at 4 weeks and 6/9 slides at 8 weeks. In Group 4, fat was found in 8/8 slides at 4 weeks and 3/7 slides at 8 weeks.

Epidermal thickness

In each of the four groups, the epidermal measurements of the scars were thinner at 8 weeks compared with 4 weeks after wound creation, and these differences were significant in Groups 2, 3 and 4 (Group 1, 4 weeks: 51.5 ± 14.7 μm, 8 weeks: 32.3 ± 4.9 μm, p=.053; Group 2: 4 weeks: 48.3 ± 11.8 μm, 8 weeks: 34.4 ± 7.9 μm, p=.013; Group 3: 4 weeks: 54.9 ± 12.5 μm, 8 weeks: 35.3 ± 2.9 μm, p=.0027; Group 4: 4 weeks: 56.9 ± 11.5 μm, 8 weeks: 32.5 ± 8.6 μm, p=.006) (Figure 5). However, there were no significant differences between the groups in epidermal thickness after wound creation.

Number of blood vessels

In each of the four groups, the number of blood vessels per field (0.08 mm^2) observed at 8 weeks after wound creation was significantly lower than that at 4 weeks after wound creation (Group 1: 4 weeks: 4.5 ± 0.7, 8 weeks: 2.8 ± 0.3, p=.0036; Group 2: 4 weeks: 4.1 ± 0.6, 8 weeks: 2.8 ± 0.8, p=.015; Group 3: 4 weeks: 4.5 ± 0.7, 8 weeks: 3.0 ± 0.7, p=.0025; Group 4: 4 weeks: 3.9 ± 0.5, 8 weeks:

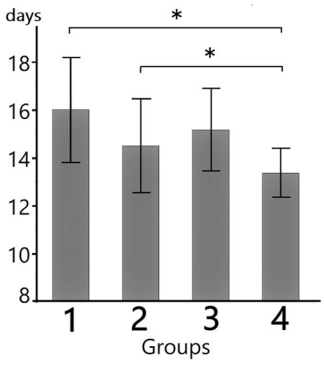

Figure 2. Comparison of ulcer wound healing time in the four study groups. The mean healing time was significantly shorter in the rats in Group 4, who received a combination of fat injection and surface grafting, compared with the Group 1 controls, whose ulcers were covered using wound dressing materials and staples only (p=.0005), and Group 3, who received surface grafts only (p=.023). Data shown as mean ± standard deviation. *$p<.05$.

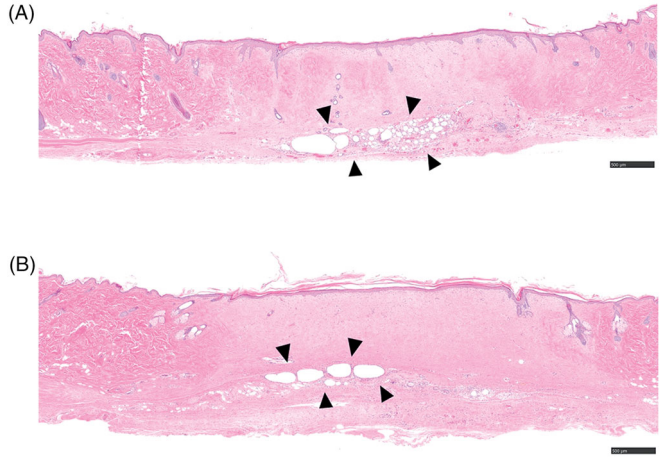

Figure 4. Representative hematoxylin-eosin-stained skin sections obtained 4 and 8 weeks after irradiation and wound creation in rats treated with fat grafted onto the surfaces of the ulcers (Group 3). Fat (indicated with arrowheads) was observed in the scar dermis at 4 weeks (A) and remained at 8 weeks (B). The bars are 500 μm.

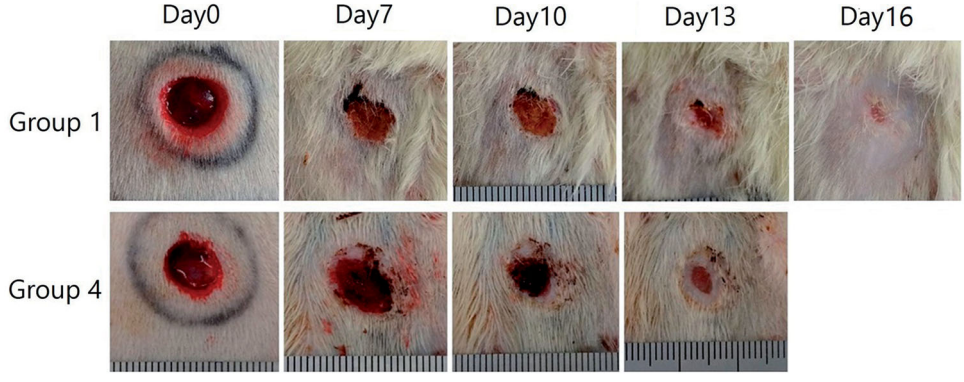

Figure 3. The closure of radiation-induced ulcer wounds over time. Representative wounds from Groups 1 and 4 are shown. In Group 1, the ulcer wounds were covered using wound dressing materials and staples only. In Group 4, a combination of fat injection and surface grafting was employed prior to wound dressing and stapling. The wounds in Group 4 had a whitish appearance without discharge or crusting on Day 13, which we judged as epithelization. Healing occurred more slowly in Group 1, in which epithelization was not observed until Day 16.

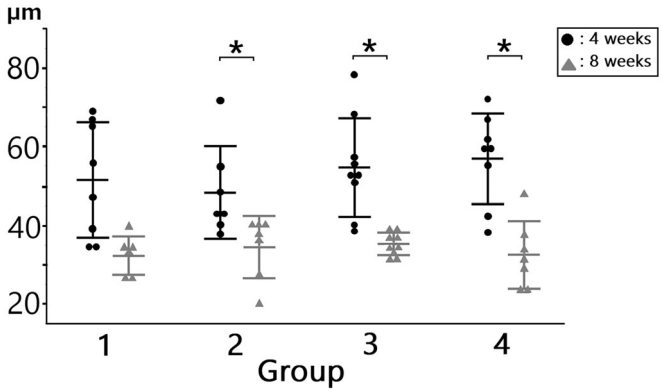

Figure 5. Epidermal thickness of scars at 4 weeks and 8 weeks after wound creation in the four groups. The 4-week data are depicted as black dots and the 8-week data as gray triangles. Horizontal bars indicate means and standard deviations. In Groups 2, 3, and 4, the epidermal thickness differences between 4 and 8 weeks were significant ($*p < .05$).

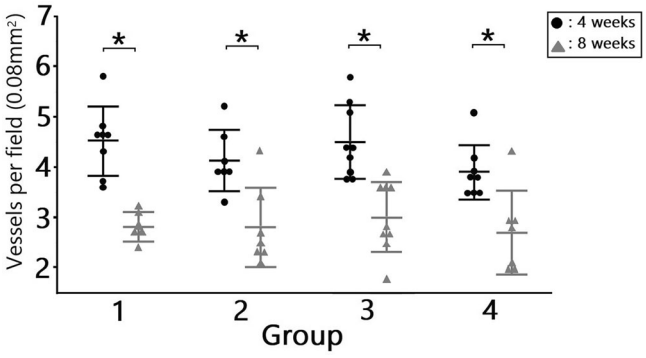

Figure 6. The numbers of blood vessels observed at 4 weeks and 8 weeks after wound creation in each group. The 4-week data are depicted as black dots and the 8-week data as gray triangles. Horizontal bars indicate means and standard deviations. In each group, the number of blood vessels was significantly lower at 8 weeks after wound creation than at 4 weeks. The vertical line of the graph shows the number of vessels per field (0.08 mm^2). $*p < .05$

2.7 ± 0.8, p=.014) (Figure 6). There were no significant differences between the groups in the number of blood vessels detected after wound creation.

Scar index

There was no significant difference in the overall mean scar index obtained at 4 and 8 weeks after wound creation in all four groups (4 weeks: 1.6 ± 1.7, 8 weeks: 2.3 ± 1.6). There were also no significant intergroup differences in the scar index at 4 or 8 weeks (Group 1: 4 weeks: 1.2 ± 1.2, 8 weeks: 1.3 ± 0.8, p = .722; Group 2: 4 weeks: 2.3 ± 3.3, 8 weeks: 2.5 ± 2.0, p=.902; Group 3: 4 weeks: 1.6 ± 1.2, 8 weeks: 2.6 ± 1.4, p=.233; Group 4: 4 weeks: 1.5 ± 0.6, 8 weeks: 2.5 ± 1.9, p=.498).

Discussion

Fat grafting is one of the treatments used for non-healing ulcers, which include radiation-induced ulcers. The most commonly used fat-grafting technique is the injection method. Our study has demonstrated that a combination of fat injection around an ulcer and fat grafting onto the surfaces of the same ulcer effectively accelerated the healing of radiation-induced ulcers in rats. In addition, during examinations of skin sections from rats in which fat had been grafted onto the surfaces of ulcers, fat tissue was observed within the dermis at 4 and 8 weeks after the grafting procedure.

Fat tissue that is obtained during liposuction procedures contains ASCs [4]. Therefore, the aspirated fat tissue that we used in the present study might have contained ASCs, which have been reported to differentiate into endothelial cells [10]. It was reported that skin perfusion decreased after radiation exposure in mice [11]. ASCs have also been reported to secrete cytokines, including angiogenic and antiapoptotic factors, vascular endothelial growth factor, hepatocyte growth factor and transforming growth factor-beta. In addition, ASC-conditioned culture medium can contain keratinocyte growth factor, basic fibroblast growth factor, platelet-derived growth factor and fibronectin [12]. These factors promote connective tissue formation and epithelization. Another study reported that ASC-conditioned culture medium increased endothelial cell growth and reduced endothelial cell apoptosis [4]. The direct application of fat grafts to the surfaces of ulcers delivers fat tissue, cytokines and growth factors to ulcers, and might promote wound healing. Therefore, this method is considered to increase the effectiveness of fat injections around radiation-induced ulcers. Indeed, in this study the healing time for the rats in Group 4, who received a combination of fat injections around ulcers and fat grafts on the surfaces of ulcers, was shorter than those of the other groups.

We found fat in the dermal tissue samples of the groups in which fat was grafted directly onto the surfaces of ulcers (groups 3 and 4). According to previous animal studies, most grafted fat cells die after grafting, with the dead fat gradually absorbed and replaced by the host's living fat [13]. Dying fat tissue causes inflammation and increases macrophage infiltration. Macrophages release adipogenic factors, which accelerate endogenous fat regeneration [14,15]. The intradermal fat observed in our study might have been a mixture of dying fat and regenerating fat. Both types of fat could contribute to the acceleration of wound healing. Therefore, grafting fat onto the surfaces of ulcers might be effective against non-healing wounds.

Other studies have reported that the application of fat grafts to ulcers promotes blood vessel formation and decreases fibrosis [9]. Acute radiodermatitis causes some degree of dermal inflammation and reportedly leads to epidermal thickening and fibrosis [1,2]. Thus, epidermal thickness and the degree of fibrosis can be used as markers of acute radiodermatitis. In one study, the epidermis of scars subjected to fat grafting were significantly thinner than those of scars that were not subjected to fat grafting, and fat grafting was shown to prevent fibrosis after irradiation [2]. In the current study, we sampled scar specimens at 4 and 8 weeks after ulcers were created using a method described previously [2]. We found that the number of blood vessels was significantly lower and the epidermis was significantly thinner at 8 weeks compared with 4 weeks, which we took to indicate the amelioration of dermatitis. However, no significant intergroup differences in these findings were observed at any time point. This might be explained by the small size of the ulcerated area (8 mm diameter), and the relatively short healing time (range, 13–19 days); that is, the maximum difference was only 6 days. Thus, the differences between groups might be expected to be small at 4 and 8 weeks after wound creation.

The detrimental effects of ionizing radiation are not restricted to the irradiated cells, but extended to non-irradiated bystander cells or even distant cells. Oxidative stress has been reported to contribute to the development of ionizing radiation-induced bystander effects [16,17]. ASCs reported to secrete antioxidant

substances that are thought to protect dermal fibroblasts from oxidative damage by decreasing the number of apoptotic cells [18]. Therefore, fat that is grafted onto the surfaces of ulcers might contribute to reducing antioxidant stress and the number of apoptotic cells, which could in turn accelerate ulcer healing.

The mortality rates of groups 1 and 2 (3/9 and 2/9, respectively) were relatively high. While the exact cause of death was not determined, most of the deaths occurred several hours after the administration of general anesthesia. The inadvertent injection of anesthesia drugs into blood vessels and organs might have caused the deaths.

In the current study, there were no obvious signs of infection around the ulcers at any point during the study. The ulcers were as small as 0.5 cm^2, and the amount of fat grafted onto the surface of each was 0.02 mL. It is possible that larger amounts of fat grafted onto the surface of larger ulcers could create an infection risk due to the lack of blood flow through the grafted fat. A future study involving larger ulcers will be required to examine this issue.

In conclusion, we have demonstrated that grafting fat onto the surfaces of radiation-induced ulcers is a simple technique that does not increase the invasiveness of the standard treatment of fat injection. In addition, fat grafting is a feasible treatment option for non-healing ulcers. The combination treatment involving the grafting fat onto the surfaces of ulcers and injection of fat around ulcers might also be effective against other types of non-healing ulcers, such as diabetic foot injuries and critical foot ischemia.

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Disclosure statement

The authors report that no conflicts of interest exist.

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ORCID

Michiko Fukuba http://orcid.org/0000-0001-9444-366X

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