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Studies in Fat Grafting: Part III. Fat grafting irradiated tissue: Improved skin quality and decreased fat graft retention

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Abstract

Background—Following radiation therapy, skin becomes fibrotic and can present a difficult problem for reconstructive surgeons. There is an increasing belief that fat grafting under radiated skin can reverse the damage caused by radiation. The present study evaluated the effect of fat grafting on irradiated skin, along with fat graft quality and retention rates in irradiated tissue.

Methods—Nine adult Crl:NU-Foxn1^{nu} CD-1 mice received 30 Gy external beam radiation of the scalp. Four weeks following radiation, scalp skin from irradiated and non-irradiated mice was harvested and compared histologically for dermal thickness, collagen content, and vascular density. Human fat grafts were then injected in the subcutaneous plane of the scalp. Skin assessment was performed in the irradiated group at 2 and 8 weeks post-grafting, and fat graft retention was measured at baseline and every 2 weeks up to 8 weeks post-grafting using micro-CT. Finally, fat graft samples were explanted at 8 weeks, and quality scoring was performed.

Results—Fat grafting resulted in decreased dermal thickness, decreased collagen content, and increased vascular density in irradiated skin. CT analysis revealed significantly decreased fat graft survival in the irradiated group when compared to the non-irradiated group. Histological scoring

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of explanted fat grafts demonstrated no difference in quality between the irradiated and non-irradiated groups.

Conclusions—Fat grafting attenuates dermal collagen deposition and vessel depletion characteristic of radiation fibrosis. Although fat graft retention rates are significantly lower in irradiated than non-irradiated tissue, the quality of retained fat between the groups is similar.

Keywords

Fat grafting; fat transfer; radiation fibrosis; dermatitis; fat retention; skin damage

Introduction

Soft tissue augmentation with fat grafting has rapidly become a popular approach to treat contour deformities in both cosmetic and reconstructive plastic surgery.(1–4) Unfortunately, retention rates of transplanted fat remain highly unpredictable, with reported values varying widely.(5–7) Importantly, successful fat grafting relies on the principle of delivering small aliquots of fat to a well-vascularized recipient site.(8–10) Despite this, fat grafting is being done with increasing frequency in scarred surgical fields and irradiated mammary or head and neck tissue that are known to be ischemic.(11–14) In these settings, however, surgeons have noted improved quality of skin following fat grafting.(14, 15)

In contrast to observational and anecdotal evidence, laboratory animal studies allow investigators to identify specific changes occurring in fibrotic skin following fat grafting.(16, 17) Cellular mechanisms and mediators involved in skin salvage after fat grafting, though, remain poorly understood.(14, 16, 17) Furthermore, prior investigations have yet to evaluate the effect of prior cutaneous radiation exposure on fat graft volume retention. The aim of the present study was to thus investigate the effect of fat grafting on irradiated skin in a nude mouse utilizing a novel scalp radiation model. The effect of fat grafting on cutaneous vascularity in irradiated tissue was of particular interest given conflicting reports in the plastic surgery literature.(14, 16, 17) In addition, this study aimed to investigate fat graft retention rates in the setting of radiation and the quality of those grafts, both measures that to our knowledge have not been studied previously.(18) Finally, this study served as a means of further validating our previously described method of *in vivo* fat graft volume analysis using micro-computed tomography (CT).(5)

Materials and Methods

Animal Model and Radiation Protocol

Fifteen adult Crl:NU-*Foxn1*^{nu} CD-1 immunocompromised mice were used for the experiments in this study (APLAC approval #9999). Six of the mice were used as non-irradiated controls. The remaining nine mice were treated with a total of 30 Gy external beam radiation, delivered as six fractions of 5 Gy over 12 days by a Kimtron Polaris X-Ray Generator (Oxford, CT). The mice were placed under a lead jig, allowing only exposure of the scalp to the radiation source (Figure 1).

Fat Grafting

After informed consent was obtained, abdominal and flank lipoaspirate was acquired from a healthy 41 year-old female using suction-assisted liposuction (IRB approval #2188). Isolation of fat from the oil layer and blood and debris layer was achieved through gravity separation for 30 minutes. Fat was transferred to 1 cc syringes attached to 16-gauge needles for injection within 2 hours of original harvest. Four weeks after completion of the radiation protocol, 200 μ l of fat was injected in the subcutaneous plane of the scalp of six previously irradiated mice and three non-irradiated mice.⁽⁵⁾ Injection sites were closed with 6-0 VICRYL® suture (Ethicon Inc., Somerville, NJ).

Skin Harvest

Full-thickness scalp skin was harvested from three mice 4 weeks after completion of radiation. Similarly, control scalp skin was harvested from three non-irradiated mice. Skin samples were fixed and embedded in paraffin for sectioning. Skin was harvested in the same manner from three irradiated mice 2 weeks after fat grafting and from three irradiated mice and three non-irradiated mice 8 weeks after fat grafting.

Skin Analysis

Assessment of radiation damage was carried out through hematoxylin and eosin (H&E) staining for measurement of dermal thickness, Picrosirius red staining for collagen content, and CD31 immunohistochemical staining. A Leica DM5000 B light microscope (Leica Microsystems, Buffalo Grove, IL) and an A X-Cite 120 Fluorescence Illumination system (Lumen Dynamics Group Inc., Ontario, Canada) were used for imaging.

To assess dermal thickness, nine sections were chosen randomly from each mouse at each time point. All measurements were taken with the 10x objective, and the thickest dermal measurement was recorded. For collagen content, three images were obtained from each section from each mouse at every time point using polarized light with the 40x objective. Image J (NIH, Bethesda, MD) was used to quantify staining based upon pixel-positive area per high power field using the same intensity threshold for all images.

To assess tissue vascularity, sections were immunohistochemically stained for CD31 (Abcam, Cambridge, MA). Three images were obtained from each section for each mouse at every time point using the 40x objective. Image J was used to quantify staining based upon pixel-positive area per high power field.

Computed Tomography

Micro-CT imaging was performed using a MicroCAT-II *in vivo* X-Ray micro-CT scanner (Imtek, Inc.) as previously described.⁽⁵⁾ Mice that underwent fat grafting were imaged 2 days following injection for baseline volume. The grafted mice then underwent serial imaging every 2 weeks up to week 8. Fat graft volume was assessed through three-dimensional reconstructions with cubic-spline interpolation.⁽⁵⁾ All reconstructions were performed by a single investigator (R.M.G.) to avoid inter-observer variability.

Histological Analysis of Fat Grafts

At week 8, mice that had undergone fat grafting and followed by serial CT imaging were euthanized, and fat grafts were harvested. The grafts were immediately fixed and embedded in paraffin for sectioning and H&E staining. Ten sections were chosen each from the irradiated and non-irradiated groups for histological analysis and scoring. Bright-field images were obtained with a 10x objective. Four blinded, independent investigators assessed the fat grafts according to a previously published protocol.(18) Each investigator evaluated integrity (assessed by presence of intact, nucleated fat cells), cyst/vacuoles, inflammation, and fibrosis.

Statistical Analysis

Data are presented as means and one standard deviation is depicted by error bars. StatPlus software (AnalystSoft Inc.) was used to perform two-sample t-tests for comparison of means, and for histological scoring, a Mann-Whitney test was performed. A * p -value < 0.05 was considered significant.

Results

Fat Grafting Attenuates Skin Fibrosis and Improves Vascularity in Irradiated Skin

Although gross changes such as skin necrosis and ulceration were not observed, cutaneous radiation damage was demonstrated histologically. The irradiated group demonstrated significantly thicker dermis (230.42 ± 25.96 microns) than the control group (182.88 ± 10.89 microns; * p < 0.05) (Figure 2). In addition, Picrosirius red staining revealed significantly more collagen in the irradiated group (443334.57 ± 65390.19 red pixels) when compared to the control group (216247.59 ± 43202.48 red pixels; * p < 0.05) (Figure 3). Finally, irradiated skin at baseline was found to have lower vascular density, with the number of red pixels measured by CD31 immunohistochemical staining significantly lower than control non-irradiated skin (non-irradiated 11315.97 ± 7151.6 , irradiated 749.06 ± 1118.2 ; ** p < 0.01) (Figure 4).

After confirming cutaneous damage in the irradiated group, both irradiated and non-irradiated mice underwent fat grafting. Dermal thickness measured at both 2 weeks (163.11 ± 9.43 microns) and 8 weeks (167.49 ± 1.09 microns) post-fat grafting in the irradiated group was significantly decreased when compared to baseline (both * p < 0.05) (Figure 2). Importantly, there was no statistically significant difference between dermal thickness in the control non-irradiated, non-fat grafted group and both week 2 and week 8 irradiated, fat grafted groups. Given the possibility that dermal thinning observed in the irradiated group could be due to a tissue expansion effect of the fat graft on the scalp skin, the dermal thickness of non-irradiated mice 8 weeks after fat grafting was measured. No dermal thinning was observed in this group, and there was no significant difference between dermal thickness in irradiated, fat-grafted mice at two and eight weeks and non-irradiated, fat grafted mice 8 weeks post-fat grafting (Figure 2).

Irradiated mice demonstrated a progressive decrease in skin collagen content measured by Picrosirius red staining following fat grafting. This difference was not statistically

significant at 2 weeks (326287.82 ± 31203.11 red pixels, $p > 0.05$) but reached statistical significance by 8 weeks post-fat grafting (253604.39 ± 18058.94 red pixels, $*p < 0.05$) (Figure 3). As observed with changes in dermal thickness, there was no observed difference in collagen content between the week 8 irradiated, fat grafted group and non-irradiated, non-fat grafted baseline control ($p > 0.05$) (Figure 3).

CD31 immunohistochemical staining resulted in an increase in staining in the irradiated groups following fat grafting (week 2: 3518 ± 1851.29 red pixels and week 8: 2571 ± 1786.75 red pixels). Week 2 vascular density was slightly higher than week 8, but the difference between the groups was not statistically significant. Although vascularity was significantly higher than that observed in irradiated skin at baseline ($**p < 0.01$ at week 2 and $*p < 0.05$ at week 8), vascularity in the irradiated, fat grafted mice at all time points remained significantly lower than non-irradiated, non-fat grafted skin (Figure 4).

Decreased Fat Graft Retention Following Radiation

While there was no significant difference in fat graft volume retention between irradiated and non-irradiated mice at weeks 2 and 4, by week 6, fat graft volume was significantly less in irradiated mice ($*p < 0.05$) (Figure 5A and B). This was similarly noted at week 8, with fat graft retention significantly lower in the irradiated group ($55.22\% \pm 2.89\%$) when compared to the non-irradiated group ($68.17\% \pm 4.12\%$; $*p < 0.05$).

Retained Fat Grafts Demonstrate Normal Quality in Irradiated Tissue

Assessment of H&E stained sections of fat explanted from irradiated and non-irradiated mice 8 weeks post-fat grafting resulted in no difference in scoring for all fat characteristics. On a scale of 0–5 (with 0 indicating “absence” and 5 indicating “extensive presence”), fat grafts in the irradiated group were given a mean score of 4 ± 0.16 for integrity. The non-irradiated group received a score of 4.35 ± 0.86 . Three factors – presence of cyst/vacuoles, inflammation, and fibrosis – were used as measures of the degree of damage to the adipocytes and also resulted in similar scores between the two groups (Cyst/Vacuoles: XRT 1.20 ± 0.19 and no XRT 1.18 ± 0.20 ; Inflammation: XRT 0.93 ± 0.15 and no XRT 1.28 ± 0.19 ; Fibrosis: XRT 1.15 ± 0.18 and no XRT 1.10 ± 0.20) (Figures 5C and 4D).

Discussion

Skin damage following irradiation is characterized by early erythema and ulceration followed by fibrosis with thickening of the dermis and subcutaneous tissue and tissue ischemia due to hypovascularity.(19, 20) Multiple pathways controlling dermal homeostasis become dysregulated, the most studied of which is the transforming growth factor beta 1 (TGFβ1)/Smad pathway.(21–23) Immediately following irradiation, an inflammatory response occurs in the skin with cellular recruitment. These inflammatory cells, along with native endothelial cells, fibroblasts, and epidermal cells, begin secreting TGFβ1, which activates Smad signaling intermediates leading to increased production of collagen and other extracellular matrix (ECM) components.(21, 22, 24–27) The ECM thus becomes abnormal in both quality and quantity, and the processes of regulatory feedback that control matrix production and degradation are disrupted.(21) In addition to its action through the Smad

pathway, TGF β 1 also recruits and stimulates myofibroblasts, the cells responsible for production of collagen and metalloproteinases.(28, 29) Interestingly, in human mammary skin of breast cancer patients treated with adjuvant radiation, upregulated gene expression for collagen types I and III and TGF β 1 has been demonstrated up to 20 years post-radiotherapy.(21) Therefore, the fibrotic cascade may persist long after the radiation source is removed.(27)

Paralleling our findings in this study, Thanik et al. showed progressive dermal thickening with increased collagen content, measured by H&E and Picrosirius red staining, following radiation to dorsal back skin in mice.(23) The authors reported dermal thickening at 2 weeks post-radiation that progressively increased up to their 6 week end-point. In addition, they also found greater Smad3 expression measured by immunohistochemical staining of irradiated skin specimens, indicating activation of the TGF β 1/Smad pathway that contributes to ECM dysregulation.(23)

In addition to accumulation of collagen, radiation fibrosis is characterized by loss of the microvasculature and subsequent hypoxia. Within hours of radiation exposure, leukocyte infiltration of vessels and fibrin plugs have been shown in rodent models.(27) Similarly, endothelial swelling and hyperplasia and fibrin plugs have been shown to obliterate capillaries early after radiation in human skin. This, in conjunction with perivascular fibrosis, ultimately leads to hypoperfusion.(27) As blood flow decreases, tissue oxygen tension is thus lowered, and hypoxia has been shown to also induce tissue fibrosis through increased collagen type 1 alpha 1 (COL1A1) expression.(30) Therefore, in addition to other pathways that stimulate fibrosis, collagen production is upregulated in response to the hypoxic state created in irradiated tissue after vessel loss.

Laser Doppler imaging has been used as a surrogate measure of vessel density in studies of radiation and thermal cutaneous injury in mice.(17, 23) Decreased blood flow observed with laser Doppler was accompanied by histological findings of vessel encasement.(23) Interestingly, flow improved when animals received fat grafts beneath injured skin, and skin harvested from the fat grafted animals also demonstrated increased vascular endothelial growth factor (VEGF) and stromal cell-derived factor 1 expression as measured by enzyme-linked immunosorbent assay and polymerase chain reaction.(17) This increased expression of vasculogenic factors was accompanied by decreased expression of COL1A1 along with matrix metalloproteinase 9, TGF β , and tissue inhibitor of metalloproteinases 1, all known inducers of fibrosis.(17) Similarly, Sultan et al. demonstrated decreased collagen deposition, as measured by Picrosirius red staining, in animals with cutaneous burns and radiation fibrosis after fat grafting.(17)

Our findings of thickened dermis, increased Picrosirius red staining, and decreased CD31 staining in the irradiated group when compared to non-irradiated mice at baseline are consistent with late radiation skin toxicity. However, as early as 2 weeks post-fat grafting, irradiated mice demonstrated significant dermal thinning that persisted to the 8-week time point. This was accompanied by decreased Picrosirius red staining, also beginning at 2 weeks. Of note, Mojallal and colleagues did observe dermal thickening following fat grafting in mice, but their study evaluated the response of normal skin and not radiation

damaged skin to injected fat.(31) Also importantly, dermal thinning is a known consequence of tissue expansion, and the injected fat graft may have expanded the overlying scalp skin to some degree.(32) However, non-irradiated skin after fat grafting did not undergo dermal thinning; therefore, dermal thinning observed in the irradiated group cannot be attributed to expansion alone. Active interruption of the cellular processes normally responsible for radiation fibrosis is further supported by the observed increase in CD31 endothelial staining.

Like Sultan et al. who investigated the effect of grafting on irradiated skin in a mouse model, the present study also found decreased dermal collagen content and thickening following fat grafting, consistent with improvement in radiation damaged skin.(16) However, while Sultan et al. observed pathologic dermal thickening only in animals that received a minimum of 40 Gy of radiation delivered in a single dose to the animal's dorsum, we noted thickening with a lower dose of 30 Gy delivered in 6 fractions to the scalp.(16) The radiation delivered in this present study was selected from a range based on the maximal sublethal amount tolerated by our mice. This can be highly variable based on mouse strain, and we selected an immunocompromised mouse to minimize inflammatory response to human fat as compared to their study which employed wild-type FVB mice. One final difference between the Sultan study and the present one is reported tissue vascularity. Following radiation, Sultan and colleagues noted early increased vascular density which progressively decreased following fat grafting.(16) It is interesting to note, however, that the same group reported the opposite finding with burn scars, where fat grafting was reported to increase vascularity.(17) In addition, Thanik and colleagues reported decreasing skin perfusion following radiation in mice, and clinical studies have shown higher vascular network density following fat grafting to irradiated host sites.(14, 23) Our data are consistent with these later observations, as we noted improved vascularity in fibrotic tissue treated with fat grafting.

Although there is growing evidence that fat grafts attenuate collagen deposition and dermal thickening and enhance angiogenesis/vasculogenesis, these processes remain poorly understood. One proposed mechanism for skin salvage is based on the fact that fat grafts are known to harbor adipose-derived stromal cells which may contribute to new vessel formation or elaborate a number of additional growth factors such as fibroblast growth factor, insulin-like growth factor, VEGF, and platelet-derived growth factor.(7, 33–36) Therefore, delivery of these cells may interrupt the cellular cascades that remain chronically activated in irradiated tissue.

Despite the improvement in skin quality observed with fat grafting in irradiated tissue, decreased fat graft retention was observed in irradiated animals when compared to non-irradiated controls. The difference between the groups was first noted at 4 weeks post-grafting and reached statistical significance by week 6. After grafting, transplanted fat exhibits three zones of healing, as described by Eto et al.(37) The central necrotic area is lost while the peripheral surviving area and intermediate regenerating area contribute to the final surviving graft volume.(37) In their *in vivo* mouse model, adipocyte cell death was observed in the first few days after grafting. By day 7, graft volume stabilized, and regeneration began. Before vascular ingrowth occurs, fat grafts in irradiated and non-irradiated tissue are both subject to ischemic conditions. But because irradiated tissue provides a poorer

vascularized bed during early fat graft incorporation, this likely contributed to lower ultimate retention rates in our study. By 8 weeks, however, fat graft remodeling has likely stabilized, and we noted the quality of retained fat in both irradiated and non-irradiated tissue was similar. Finally, it is important to note that our fat graft retention rates observed in non-irradiated controls were comparable to previous reports, thereby supporting the validity of our approach.(5)

Conclusions

Delivery of 30 Gy of radiation to the scalp of nude mice results in skin damage (i.e. fibrosis and pathologic dermal thickening) consistent with radiation fibrosis. Reduced dermal thickening and collagen deposition were noted while vascularity was enhanced in irradiated tissue following fat grafting, all consistent with improved skin quality. Although lower rates of fat volume retention were observed in irradiated animals, the quality of remaining fat was similar to that observed in non-irradiated controls. While these findings begin to elucidate the relationship between irradiated tissue and fat grafts, the processes involved in fat graft incorporation and remodeling must still be further investigated for development of interventions that will enhance retention and long-term outcomes in both irradiated and non-irradiated tissue.

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Financial Disclosure and Products Page

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Figure 1.
Delivery of radiation to mice. (A) Photograph of Kimtron Polaris X-Ray Generator. (B) Photograph of lead jig allowing only exposure of the scalp to radiation. Semi-transparent mouse shown beneath the lead jig.

Figure 2.

Dermal thickening following radiation and fat grafting. Representative H&E stained sections (above) demonstrated significantly thickened dermis in scalp four weeks after completion of irradiation and prior to fat grafting (second box above and bar below labeled “XRT”) when compared to both non-irradiated, non-fat grafted animals at baseline (first box and bar labeled “No XRT”; $*p < 0.05$). Irradiated skin prior to fat grafting was also significantly thicker than irradiated scalp at 2 and 8 weeks post-fat grafting (third and fourth boxes and bars labeled “XRT+FAT W2” and “XRT+FAT W8”; both $*p < 0.05$). Non-irradiated skin did not demonstrate dermal thinning 8 weeks following fat grafting (fifth box and bar labeled “No XRT+FAT W8”) and was not significantly different from irradiated scalp with fat grafting at 8 weeks.

Figure 3.

Radiation and fat grafting effects on collagen content. Picrosirius red staining (above) demonstrated significantly higher density of positive-stained collagen (red) in skin four weeks after completion of irradiation and prior fat grafting (second box above and bar below labeled “XRT”) than non-irradiated skin with no fat grafting (first box above and bar labeled “No XRT”; $*p < 0.05$). Irradiated skin 2 weeks post-fat grafting demonstrated lower levels of staining (third box and bar labeled “XRT+FAT W2”), but this did not reach statistical significance until 8 weeks post-fat grafting (fourth box and bar labeled “XRT+FAT W8”; $*p < 0.05$).

Figure 4.

CD31 Immunohistochemical staining (red) with DAPI counterstain (blue) following radiation and fat grafting. Irradiated skin both 2 and 8 weeks post-fat grafting (third and fourth boxes above and bars below labeled “XRT+FAT W2” and “XRT+FAT W8”, respectively, demonstrated significantly higher vascular density (** $p < 0.01$ and * $p < 0.05$, respectively) measured by CD31 staining when compared to irradiated tissue prior to fat grafting (second box and bar labeled “XRT”). However, the vascular density in the 2 and 8-week irradiated skin samples did not reach normal, non-irradiated control levels represented by the first box above and bar labeled “No XRT” below.

Figure 5.

Fat graft volume retention. (A) Representative micro-CT volume reconstruction of fat graft in irradiated (left) and non-irradiated (right) scalp at week 8. (B) Significantly higher levels of fat graft retention were observed in non-irradiated mice when compared to the irradiated group at both 6 and 8 weeks post-fat grafting (both $*p < 0.05$). (C) However, no statistical difference in quality of explanted fat grafts at 8 weeks between irradiated and non-irradiated groups was noted. (D) Representative H&E stained sections of explanted fat at week 8 from irradiated (left) and non-irradiated (right) mice that were used for quality scoring analysis.