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Fat Grafting into Younger Recipients Improves Volume Retention in an Animal Model

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Abstract

Background: Soft tissue deficits associated with various craniofacial anomalies can be addressed by fat grafting, although outcomes remain unpredictable. Furthermore, consensus does not exist for timing of these procedures. While some advocate approaching soft tissue reconstruction after the underlying skeletal foundation has been corrected, other studies have suggested earlier grafting may exploit a younger recipient niche that is more conducive for fat graft survival. As there is a dearth of research investigating effects of recipient age on fat graft volume retention, this study compared the effectiveness of fat grafting in younger versus older animals through a longitudinal, *in vivo* analysis.

Methods: Human lipoaspirate from three healthy female donors was grafted subcutaneously over the calvarium of immunocompromised mice. Volume retention over 8 weeks was evaluated using micro-computed tomography in three experimental ages – 3-weeks old, 6-months old, and 1-year old. Histology was performed on explanted grafts to evaluate graft health and vascularity. Recipient site vascularity was also evaluated by confocal microscopy.

Results: Greatest retention of fat graft volume was noted in the youngest group compared to both older groups ($*p < 0.05$) at 6 and 8 weeks following grafting. Histological and immunohistochemical analyses revealed that improved retention in younger groups was associated with greater fat graft integrity and more robust vascularization.

Conclusion: Our study provides evidence that grafting fat into a younger recipient site correlates with improved volume retention over time, suggesting that beginning soft tissue reconstruction with fat grafting in patients at an earlier age may be preferable to late correction.

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Keywords

Fat Grafting; Recipient Age; Juvenile Mouse; Soft Tissue

Introduction

Soft tissue defects in the craniofacial region can arise from trauma, tumor removal, infection, congenital disorders, and connective tissue disease, and present a common reconstructive challenge for plastic surgeons. Autologous fat transfer has become an increasingly prevalent method to restore volume in soft tissue defects, owing to its high safety profile, low donor site morbidity, and relative abundance (1–5). However, fat grafting remains suboptimal, with variable volume retention reported (6–10). Furthermore, optimal timing of these procedures in the pediatric population has not yet been standardized.

Traditionally, reconstruction of large defects has begun with restoration of underlying bony structure, followed later by soft tissue correction, using a combination of flaps, synthetic fillers, and/or autologous fat transfer (11–14). However, some surgeons suggest that beginning autologous fat transfer earlier may take advantage of the higher reparative potential of a younger niche, which could produce more stable volume outcomes. Tanna et al. found that serial fat grafting, started early in the process of reconstruction, can lead to volumetric results comparable to those observed with use of flaps, along with the added benefit of lower complications and lower overall operating time (15). Furthermore, a study examining surgical timing for reconstruction in Poland syndrome found that starting autologous fat transfer earlier, during the period of growth, allowed for greater body image stabilization with more frequent, but less invasive procedures (16). The principle that younger recipient sites may retain volume better has also been supported by the finding that overcorrection during autologous fat transfer, a common technique to counter unpredictable volume loss in adults, can in some pediatric cases lead to the complication of “sustained overcorrection,” resulting in larger than intended volumes (17).

While the literature suggests fat grafting at earlier ages may be reasonable, there is a dearth of exploration into how age of the recipient specifically influences effectiveness of fat grafting. Fat graft retention might be improved by grafting into younger recipient sites, which would be particularly relevant for reconstructions that require multiple procedures over the course of months to years. To this end, we employed a longitudinal *in vivo* model to measure how fat graft volume retention differs in mice groups of various ages. Histology was also performed on explanted fat grafts to explore vascularization of the graft and compare how the architecture of the fat graft changes with age of the recipient site.

Methods

Fat Grafting

All studies were conducted in accordance with Stanford University animal use guidelines (APLAC Protocol #31212). Crl:Nu-*Foxn1*^{nu} immunocompromised mice (Charles River Laboratories; Wilmington, MA) were used for *in vivo* analysis to minimize immunologic

response to human adipose tissue (18). Three mouse age groups (n=15 per group) were chosen to reflect different clinical ages: Group A = 3-weeks old (pre-adolescent), Group B = 6-months old (middle-age adult), and Group C = 1-year old (geriatric). Human lipoaspirate from three healthy female donors with no other medical comorbidities, ages 39- to 45-years old with BMI ranging from 26 to 29, was collected under an approved IRB protocol #2188. Lipoaspirate was allowed to settle for 15 minutes, and then oil and blood layers were removed by vacuum aspiration. The remaining fat layer was centrifuged at 1300 rcf for 3 minutes at 4°C. Remaining oil and blood was again removed and the resultant fat was transferred into 1cc syringes for injection. A 14-gauge Luer-lock cannula was used to create a subcutaneous tunneled pocket over the calvarium of each mouse, and 150 microliters of fat was subsequently injected with skin sutured closed (Figure 1).

Fat Graft Volume Retention

Fat graft volume was measured two days after fat graft injection for baseline volume measurements and then every two weeks over 8 weeks using a MicroCAT-II *in vivo* X-Ray micro-CT scanner (Imtek, Inc.; Knoxville, TN). Cubic-spline interpolation was used to reconstruct three-dimensional images of grafts and measure volume changes over time (18), as previously described. All reconstructions were performed by a single investigator (N.N.C.) to avoid inter-observer variability.

Fat Graft Histological Analysis

Three fat grafts were explanted at two, four, six, and eight weeks from each age group for histological analysis. Samples were fixed, embedded in paraffin, sectioned, and stained with hematoxylin and eosin (H&E). Bright-field images were obtained with a 10x objective. Five sections per graft were analyzed at each time point by five blinded, independent reviewers, who assessed metrics of fat health/tissue integrity, as well as injury including formation of cysts/vacuoles, inflammation, and fibrosis, as previously described (19, 20). Sections were also stained using fluorescent immunohistochemical anti-CD31 antibody (Ab28364 1:100 dilution; Abcam, Cambridge, MA) to assess for vascularity. Fluorescence was quantified using ImageJ analysis.

Skin Staining and Confocal Imaging

Skin biopsies from three animals in Groups A, B, and C, were obtained prior to fat graft placement. Each sample was then stained with Isolectin endothelial cell stain (ThermoFisher Scientific; Waltham, MA) (20 µl in 2 ml PBS for a 1:100 dilution) and FITC anti-mouse CD31 antibody (BioLegend; San Diego, CA) (1:100 dilution) for one hour at room temperature. After a brief PBS wash, the samples were then incubated at room temperature in a 1:2000 dilution of Hoechst 33342 (ThermoFisher Scientific) stain in PBS for 30 minutes. The samples were finally washed twice with PBS and whole-mounted to glass microscope slides, as adapted from the protocol described by Berry et al. (21). Laser scanning confocal microscopy was performed using a Leica TCS SP8 X confocal microscope (Leica Microsystems; Wetzlar, Germany) with an objective lens (10× HC PL APO, air, N.A. 0.40). Three-dimensional volume rendering of z-stacks was performed on Imaris (Bitplane AG; Zurich, Switzerland) and quantification was performed using the Isosurface Rendering Statistics tool.

Statistical Analysis

Data are presented as medians $\pm$ range. Multiple group comparisons were performed using a nonparametric Kruskal-Wallis with post-hoc Dunn's testing. All analyses were performed using GraphPad Prism (GraphPad Software, La Jolla, CA). A p -value <0.05 was considered statistically significant.

Results

Fat Graft Volume Retention

Three-dimensional volumetric analysis of fat grafts demonstrated that younger recipient age correlated positively with fat graft retention over 8 weeks (Figure 2). Results were statistically significant at both 6- and 8-week time points (** $p < 0.01$). At 6 weeks, the youngest group, Group A, demonstrated the greatest volume retention (79.7%, range 78.6 to 83.4%), followed by Group B (56.9%, range 52.7 to 66.2%), and finally Group C (45.9%, range 39.2 to 51.4%), the eldest group. By the end of the study at 8-weeks, Group A continued to retain higher fat graft volumes (73.3%, range 68.7 to 76.1%), again followed by Group B (45.3%, range 42.5 to 51.1%), and finally Group C (35.4%, range 30.7 to 39.9%), which demonstrated the poorest volume retention.

Fat Graft Histological Analysis

Histological examination of explanted grafts demonstrated greatest overall health in the youngest group (Figure 3) (See Figure, Supplemental Digital Content 1, which shows Histological assessment of graft architecture and health at weeks 2, 4, and 6 (n=3 grafts per group per time point, five sections analyzed per graft, five blinded scorers). (A) Representative H&E stained sections of fat grafts from different age groups at weeks 2, 4, and 6 (10 $\times$ magnification, scale bar = 200 μ m). (B) Histology scoring for architectural characteristics of fat grafts from each group at weeks 2, 4, and 6, with fat graft median and range for health/integrity on the vertical axis, and median and range for injury score comprised of cyst/vacuole formation, inflammation, and fibrosis on the horizontal axis (** $p < 0.01$), [INSERT HYPER LINK](#)). Integrity scores in Group A were significantly greater than Groups B and C at all time points (** $p < 0.01$). Scores for tissue integrity were lowest in the oldest group, which also had the highest injury scores for inflammation, cysts or vacuoles, and fibrosis at each time point. Within a single age group, it was found that health/integrity of the tissue architecture generally decreased between weeks 2 and 8 and, as expected, injury scores increased due to formation of cyst and vacuoles, as well as fibrosis. These findings were observed in each group.

On immunohistochemical assessment for CD31, it was found that fat grafts in younger recipients (Group A) had significantly greater vascularity at each time point than those in Group B or Group C, measured by anti-CD31 antibody fluorescence per high-power field (** $p < 0.01$) (See Figure, Supplemental Digital Content 2, which shows Immunohistochemical staining and quantification of CD31 at weeks 2, 4, and 6 (n=3 grafts per group per time point. (A) Representative CD31 stained sections of fat grafts from different age groups at weeks 2, 4, and 6 (20 $\times$ magnification, scale bar = 100 μ m). (B) Quantification of staining with percent CD31 (red) fluorescence per high-power field

(median and range) for each age group (** $p < 0.01$), [INSERT HYPER LINK](#), indicating higher levels of vascularization in the youngest groups that was consistent over 8 weeks. As expected, fat grafts in Group C had the lowest amount of vascularity at each time point over 8 weeks.

Recipient Site Histological Analysis

To evaluate for differences in the recipient site soft tissue, skin samples were obtained from animals in each group and Isolectin was used as a vascular stain. Three-dimensional confocal microscopy with isosurface rendering of vasculature revealed increased staining at the recipient site samples adjacent to where fat grafts would be placed from younger animals (Group A) compared to animals from either older group (Groups B and C) (Figure 4). Quantification of three-dimensional vascular density demonstrated significantly greater amount of vascularity in Group A relative to Groups B and C (** $p < 0.01$).

Discussion

Bone and soft-tissue deficiencies may contribute to facial asymmetry seen in a wide range of congenital and acquired craniofacial disorders. Multiple, staged procedures are frequently needed, and can be performed beginning as early as the neonatal period and extend into adult-hood. But despite severe asymmetry, many surgeons have advocated delaying soft tissue correction until after the facial skeletal deficiency has been addressed (22–25). By first establishing a stable skeletal foundation, soft tissue correction may then be considered as a “finishing touch.” This approach was further supported by the notion that in many instances, early soft tissue correction was not thought to alter the underlying pathophysiology and natural course of the disease (26–29).

With the popularity of autologous fat grafting increasing for correction of facial asymmetry over the last decade, however, other authors have begun to advocate for earlier intervention, especially in children with significant asymmetry who, without correction, may be subject to psychosocial issues during development. In comparing outcomes for serial fat grafting to correction of soft-tissue deficiency with microvascular free tissue transfer for craniofacial microsomia, Tanna and colleagues reported that fat grafting can be safely performed in combination with other operative interventions throughout childhood, and can result in good symmetry outcomes when done before the skeletal deficiency is addressed (15). With Parry-Romberg, serial fat grafting has also been described to be effective when begun at an early age (30). Furthermore, fat grafting performed in younger children was found to be associated with greater patient/family satisfaction scores compared to patients who had fat grafting delayed until they were older (29). Finally, in patients with Treacher Collins syndrome, Konofaos et al. reported that early fat grafting at greater than six months of age can effectively improve contour and tissue quality in the orbitozygomatic area (31). Moreover, they suggested that fat grafting prior to bone reconstruction may possibly minimize subsequent bone resorption through improved pliability and vascularization of the soft tissue envelop (32).

In considering timing for fat grafting, some studies have shown variability in fat graft quality with increasing age, particularly with regard to the stromal cell population. In older patients,

unfavorable growth kinetics and differentiation/functional capacity of adipose-derived stromal cells (ASCs) have been reported (33, 34), with reduced induction of vasculogenesis in wound healing, therapeutic benefits for pulmonary fibrosis, and rescue of mouse hind-limb ischemia described for old compared to young ASCs (35–37). Age-related changes to ASCs may be particularly relevant to fat graft retention, as studies have shown stromal cells within fat to promote early re-vascularization and potentially contribute to mature adipocyte volume through direct differentiation (38–41). These findings thus argue for earlier fat grafting to enhance volume retention.

Aside from these intrinsic considerations, quality of the macroenvironmental niche into which the fat graft is placed may be of similar importance for long-term outcomes. Fat graft retention has been shown to be significantly reduced when placed into fibrotic, hypovascular recipient beds (20, 42). Relative to age, progressive changes to the vascularity and mechanical properties of soft tissue have also been well described, and these may likewise impact fat graft retention (43). Finally, it has been shown that young, but not old, extracellular matrix may preserve proliferative and self-renewal capacity of adult mesenchymal cells, possibly through increased expression of telomerase in neighboring cells or diminished expression of reactive oxygen species (44–46). These reports thus suggest that a younger macroenvironmental niche into which fat grafts are placed may be more conducive to retention of transferred fat.

In this present study, we evaluated the effects of recipient bed age on retention of human fat grafts. We observed that fat grafting into the young recipient bed improved volume retention when compared to older recipient beds. This was associated with enhanced CD31 staining and improved fat graft histology, with greater histologic quality noted in fat grafts placed into younger animals. Finally, evaluation of the recipient site with Isolectin staining and visualization by three-dimensional confocal microscopy demonstrated significantly increased vascularity in younger animals, which may promote greater early re-vascularization of ischemic fat grafts following placement. While there are undoubtedly other recipient site factors that may impact fat graft retention, these data nonetheless support beginning soft tissue correction at earlier ages, particularly in reconstructions that require multiple surgeries, such as for craniofacial microsomia, as staged procedures afford the opportunity to also perform concomitant fat grafting at younger ages.

Importantly, the findings from this investigation may have some translational limitations. Autologous fat transfer implies grafting into the same patient from which the donor fat was harvested. Therefore, it may be more appropriate to match a young recipient site with young donor fat, and investigate fat grafting outcomes within this context. However, exploring this would require sufficient samples from pediatric patients, the accessibility of which is more challenging than that of human adult fat, particularly with regard to available volume. As a result, only adult donor fat was used in this study. Nonetheless, use of only adult fat allowed for determination of recipient bed age-related effects on graft retention independent of fat graft age. Fat grafting was also done in immunocompromised mice to minimize recipient response to xenogeneic tissue. While this model has been extensively used in the literature to study human fat (18, 20, 41, 42), in the clinical setting, a normal immune/inflammatory response to autologous fat may also impact ultimate volume retention. Alternatively, other

studies have frequently employed mouse inguinal fat pads transplanted into immunocompetent recipients to more accurately reflect the immune response encountered in patients (38, 40). However, dramatic species-specific differences in adipose tissue between mouse inguinal fat and human abdominal fat would potentially limit the translatability of this approach (47–50).

Our findings may also not fully extend to all causes of soft tissue deformities, as the pathophysiology of some conditions (e.g. Parry Romberg syndrome, post-radiation atrophy) may impact the recipient site quality. Slack et al. reported decreased fat graft retention in patients with Parry Romberg compared to non-syndromic/ cosmetic patients receiving fat grafts at similar locations (30). However, whether this was due to reduced blood supply in the recipient bed or something else intrinsic to the disease process remains unknown. Experiments in our study used healthy mouse recipient tissue, and it can be argued that the outcomes observed may be contingent on the presence of normal tissue in the recipient bed. Therefore, some disease states may respond differently to fat grafting, and the role recipient age may play in these settings remains less clear. Finally, recipient ages for mice in this study may not directly translate to humans, as species specific differences undoubtedly exist in the aging process. While our data did demonstrate improved fat graft retention in younger animals, recapitulation of these findings in humans will require additional clinical studies.

Conclusion

While fat grafting has become an increasing popular modality to manage soft tissue deficits, no consensus has been reached regarding optimal timing for these procedures. Our *in vivo* results have demonstrated that placement of fat grafts into a younger recipient bed, versus an older recipient bed, is advantageous for maintenance of graft volume. Additional histologic evidence showed improved vascularization and overall health of grafts that were placed in a younger macroenvironmental niche. A more longitudinal approach to soft tissue correction starting at a younger age may thus provide benefit to the patient with more predictable results.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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GROUP A
3-weeks old

GROUP B
6-months old

GROUP C
1-year old

Figure 1.

Three age groups of immunocompromised mice (Group A = 3-weeks old, Group B = 6-months old, Group C = 1-year old) with 150 microliter fat grafts placed subcutaneously over the calvarium.

Figure 2.

Fat graft volume retention over time. (top) Representative three-dimensional reconstructed images using microcomputed tomography of fat grafts from each age group at 8 weeks post-grafting. (bottom) Median and range for fat graft volume retention (n=6 animals per group) over 8 weeks, expressed as a percentage of the baseline volume per graft, measured on post-operative day 1. (** $p < 0.01$.)

Figure 3.

Histological assessment of graft architecture and health (n=3 grafts per group per time point, five sections analyzed per graft, five blinded scorers). (top, left) Representative H&E stained sections of fat grafts from different age groups at week 8 (10× magnification, scale bar = 200 μm). (top, right) Histology scoring for architectural characteristics of fat grafts from each group at week 8, with median and range for fat graft health/integrity on the vertical axis, and median and range for injury score comprised of cyst/vacuole formation, inflammation, and fibrosis on the horizontal axis (** $p < 0.01$). (bottom, left) Representative CD31 stained sections of fat grafts (red) with DAPI (blue) counterstain from different age groups (A, B, and C) at week 8 (20× magnification, scale bar = 100 μm). (bottom, right) Quantification of staining with percent CD31 fluorescence per high-power field for each age group (** $p < 0.01$).

Figure 4.

Confocal imaging of recipient site vascularity. (above) Isosurface rendering of vasculature from confocal images obtained by whole-mounted skin harvested from animals in Groups A, B, and C and stained with Isolectin (red), Hoechst 33342 (blue), and CD31 (green), scale bar = 200 μm . (Below) Quantification of vascular density demonstrated significantly greater vascularity in Group A recipient site skin compared to Groups B and C (** $p < 0.01$).