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Studies in Fat Grafting: Part V. Cell-Assisted Lipotransfer to Enhance Fat Graft Retention is Dose Dependent

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Abstract

Background—Cell-assisted lipotransfer has shown much promise as a technique to improve fat graft take. However, the concentration of stromal vascular fraction cells required to optimally enhance fat graft retention remains unknown.

Methods—Human lipoaspirate was processed for both fat transfer and harvest of stromal vascular fraction (SVF) cells. Cells were then mixed back with fat at varying concentrations ranging from 10,000 to 10 million cells per 200 μ l of fat. Fat graft volume retention was assessed via CT scanning over 8 weeks, and then fat grafts were explanted and compared histologically for overall architecture and vascularity.

Results—Maximum fat graft retention was seen at a concentration of 10,000 cells per 200 μ l of fat. The addition of higher number of cells negatively impacted fat graft retention, with supplementation of 10 million cells producing the lowest final volumes, lower than fat alone. Interestingly, fat grafts supplemented with 10,000 cells showed significantly increased vascularity and decreased inflammation, while fat grafts supplemented with 10 million cells showed significant lipodegeneration compared to fat alone

Conclusions—Our study demonstrates dose dependence in the number of SVF cells that can be added to a fat graft to enhance retention. While cell-assisted lipotransfer may help promote graft survival, this effect may need to be balanced with the increased metabolic load of added cells that may compete with adipocytes for nutrients during the post-graft period.

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Keywords

Fat grafting; adipose derived stem cells; volume retention; cell-assisted lipotransfer

Introduction

Despite its proven efficacy as a contouring tool in both reconstructive and cosmetic surgical procedures, fat grafting remains a relatively unpredictable technique (1). Reported graft retention rates vary from 10–90% (1–3). This has led to innovations ranging from minor modifications to lipofilling as described by Coleman, to the use of automated devices designed to preserve adipose tissue/cell integrity (4, 5). Further efforts aimed at improving fat transfer outcomes have included the use of adipose-derived stromal cells (ASCs) found within the stromal vascular fraction (SVF) of mature fat. Interestingly, the number of ASCs naturally present within adipose tissue has been reported to vary from patient-to-patient, and may be linked to the varying retention rates seen among patients undergoing autologous fat transfer (6). Thus, in order to augment the effects of native ASCs and/or compensate for local deficiencies, cell-assisted lipotransfer (CAL), the technique of utilizing fat graft supplemented with additional autologous cells from the SVF, has emerged. Indeed, since its initial description by Yoshimura in 2006 (7), CAL continues to grow in popularity as a promising technique for the improvement of fat graft retention and survival.

In contrast to modifications in the method of graft harvest and strategies for injection, which may improve initial tissue and cellular viability, CAL attempts to confer a more long-lasting advantage to the transplanted adipose tissue. ASCs have the ability to differentiate into cells of various lineages, including adipocytes, osteoblasts, myocytes, and chondrocytes (8). In addition to this multilineage capacity, ASCs have also been found to secrete a variety of pro-angiogenic and anti-inflammatory factors into their local microenvironment (9, 10). This pro-angiogenic paracrine activity of ASCs, which includes secretion of vascular endothelial growth factor (VEGF), basic fibroblast growth factor, and hepatocyte growth factor (10, 11), is particularly relevant in the hypoxic setting of freshly placed grafts. In fact, there is a large body of evidence suggesting that hypoxic conditions can improve both the proliferation and angiogenic abilities of ASCs (12–16). This seemingly beneficial relationship has been assayed in the *in vivo* setting, as ASCs have been added to ischemic flaps, normal and diabetic wounds, and models of cardiac ischemia, all with encouraging results (17–20).

Given the positive effects of ASC supplementation in other ischemic *in vivo* models, it is not surprising that CAL has shown success in both animal and human studies (21–23). Notably, a recent study by Kølkle et al. served as the first randomized controlled trial to evaluate CAL (24). The study compared the efficacy of supplementing large-volume fat grafts with *ex-vivo* expanded ASCs at a concentration 2000-times greater than what is seen in normal adipose tissue (24). Though this concentration of ASCs proved effective in increasing fat graft retention, the question remains as to whether there exists an optimal amount of added cells for enhancement of fat graft take. A study by Li et al. recently attempted to address this, using both platelet-rich plasma (PRP) in addition to ASCs cultured for 24 hours (25).

Contrasting this, we have evaluated addition of freshly harvested SVF cells alone, a more translatable approach than using *ex vivo* cultured ASCs, to enhance fat graft survival. SVF is a heterogeneous cell population consisting of endothelial and endothelial progenitor cells, pericytes, fibroblasts, and immune cells in addition to ASCs (9, 26). Flow cytometry experiments have attempted to clarify the relative amounts of these populations, though reported values vary: the number of ASCs has been reported as ranging from 3–10% (9, 27), while the number of hematopoietic cells (CD45+) ranges from 9–57% (9). Unpublished data from our laboratory examining cell subpopulations of SVF has found that approximately 1–3% of isolated cells are hematopoietic, while ASCs, which we have traditionally (albeit broadly) defined as CD34+/CD31–/CD45– cells, make up 9–16% of the SVF. Looking at post-graft volumes with varying concentrations of SVF cells, we define a number of cells to be added to fat which promotes the greatest retention of volume.

Methods

Preparation of SVF-Enriched Lipoaspirate

Fresh human lipoaspirate was obtained from two healthy female donors, both 43 years old, with no other medical comorbidities, after informed consent under Stanford University Institutional Review Board approval no. 2188. Lipoaspirate was washed, and fat separated from oil and other fluids through centrifugation for 5 minutes at 500 g. Half of the specimen to be used as fat graft was then set aside on ice for 1 hour, while the remaining lipoaspirate was further processed to obtain the ASC-containing SVF, as previously described (Figure 1) (28).

SVF cells were resuspended in phosphate-buffered saline (PBS) and counted. The fat initially obtained from the lipoaspirate was then taken off ice and varying amounts of cells were then mixed with aliquots of fat. Four different groups of SVF-enriched fat grafts were prepared at concentrations of 1×10^4 , 1×10^5 , 1×10^6 , and 1×10^7 cells added per 200 μ l of fat. An additional control group was designed with fat receiving PBS alone.

Fat Grafting

SVF-enriched fat grafts were transferred to a 1cc syringe with 16-gauge needle, and injected beneath the scalps of 30 adult Crl:NU-Foxn1^{nu}CD-1 immunocompromised mice (Charles River Laboratories International, Inc., Hollister, CA) (Figure 1) (29). This procedure was performed under Stanford University Administrative Panel on Laboratory Animal Care approval no. 9999. Briefly, an incision was made in the skin and a subcutaneous tunnel was created with the needle. Fat grafts were then injected (200 μ l) in retrograde fashion (29). A total of five groups were created (n=6 mice per group), including fat grafts enriched with 1×10^4 , 1×10^5 , 1×10^6 , and 1×10^7 SVF cells per 200 μ l, and a control fat group with no additional cells.

Imaging Analysis

Micro-CT imaging was performed two days following grafting for baseline volume measurements, and subsequently repeated every two weeks for a total of eight weeks. Mice were scanned in the ventral position using a MicroCAT-II *in vivo* X-ray micro-CT scanner

(Imtek, Inc./Siemens, Munich, Bavaria), as described previously (29–31). Fat was distinguished from skin and bone by Hounsfield units, and a user-defined region of interest was established in coronal and sagittal slices. Fat volume at each time point was then measured by reconstructing a three-dimensional surface through cubic-spline interpolation, by a single, blinded observer (29). In addition, to eliminate inter-user variability, a single person performed all volume analyses (K.J.P.).

Histological Analysis

Histological analysis was performed after Week 8. Mice were euthanized and fat grafts were explanted from scalps, fixed in 10% formalin, and embedded in paraffin. 10-micron sections were stained with hematoxylin and eosin for analysis of fat graft structure. A Leica DM5000B light microscope (Leica Microsystems, Buffalo Grove, IL) at the 10× objective was used for bright field imaging. Based on a previously-published method, histological scoring was performed by four blinded observers in order to assess overall fat graft integrity (presence of intact, nucleated adipocytes), presence of cysts and vacuoles (seen in degenerating adipose tissue), level of inflammatory infiltrate (evidenced by infiltration of lymphocytes, macrophages, and other immune cells), and fibrosis (level of collagen and elastic fibers present) (31–33). This histological scoring method is established in literature, and relies on a scaling system for evaluation of each of the four parameters: 0 = absent; 1 = minimally present, 2 = minimally to moderately present, 3 = moderately present, 4 = moderately to extensively present, and 5 = extensively present (33, 34).

CD31 (PECAM-1) immunohistochemical staining (Ab28364; Abcam, Cambridge, MA) was also performed for analysis of graft vascularity. A Hoechst 33342 nucleic acid stain (Life Technologies, Green Island, NY) was used for counterstaining. Stained sections were imaged using an X-Cite 120 Fluorescence Illumination system (Lumen Dynamics Group, Inc., Ontario, Canada) at 40× magnification. CD31 staining was quantified using Image J (NIH, Bethesda, MD) based on pixel-positive area per high power field.

Statistical Analysis

Statistical analysis was performed using a one-way analysis of variance (ANOVA) for comparisons of multiple groups, with Tukey's multiple comparisons tests used for post-hoc analysis. Two-tailed Student's *t*-tests were used for direct comparisons between two groups. A *p*-value < 0.05 was considered significant. All data are presented as mean ± standard deviation.

Results

Effects of ASC-Supplementation on Fat Graft Volume Retention

CT scans taken at two-week intervals throughout the 8-week post-grafting period were reconstructed (Figure 2a). Volume analysis showed significantly decreased graft resorption (***p* < 0.01, **p* < 0.05) among grafts treated with 10,000 SVF cells compared to control fat grafts without added cells, beginning as early as two weeks post-grafting (Figure 2b). By 8 weeks post-grafting, mice receiving grafts supplemented with 10,000 cells had significantly larger (**p* < 0.05) grafts compared to all other groups, including unsupplemented fat grafts.

Furthermore, fat grafts supplemented with 10 million cells performed significantly ($*p<0.05$) worse than all groups, with the lowest volume measured at eight weeks (Figure 2c).

Effects of SVF-Supplementation on Fat Graft Architecture and Vascularity

Statistical analysis of overall histology scoring results showed no significant difference in the amount of fibrosis between the fat graft groups, however fat grafts supplemented with 10 million SVF cells had the most inflammation and fibrosis noted (Figure 3). SVF cell supplementation with 10 million cells significantly decreased final fat graft integrity compared to fat alone ($*p<0.05$) and compared to supplementation with 100,000 cells ($*p<0.05$). Finally, significantly more cysts and vacuoles ($*p<0.05$) were seen in grafts receiving 10 million cells compared to grafts of fat alone (Figure 3).

CD31 immunostaining showed a highly significant ($***p < 0.001$) increase in vascularity of the group receiving fat grafts supplemented with 10,000 cells. Conversely, mice receiving supplementation with 10 million cells had grafts with significantly ($*p<0.05$) decreased vascularity compared to mice receiving grafts of fat alone (Figure 4).

Discussion

In spite of decades of surgical innovation, fat grafting remains a relatively inexact, imperfect science. While CAL continues to gain momentum as a technique for the enhancement of fat graft retention, many questions remain to be answered before it can be routinely used in clinical practice. One of the largest questions concerns how many cells are adequate for maximum graft volume retention, an important factor that must be taken into account during surgical planning. Considering a reported ratio of 50,000 ASCs per milliliter of lipoaspirate (27), the amount of fat a surgeon performing CAL should set aside for harvest of SVF could conceivably vary by orders of magnitude, depending on the number of cells he or she wishes to add and the volume of fat to be injected.

In order to address this question, we utilized a murine model of xenografting and high-resolution CT scanning in order to precisely estimate fat graft volumes (29). By using an immunocompromised mouse strain, we were able to evaluate the interactions between patient-matched human fat and SVF cells. Though the mice used were still to some degree immunocompetent and thus could not perfectly replicate conditions of human fat autografts, they are commonly used in xenograft experiments (35, 36), and represented a natural choice for our study. This model has been found to yield reproducible results and provides an accurate real-time assessment of small-volume fat transfer (29, 31, 37).

Utilizing this model, we have also found that when it comes to small-volume CAL, “less is more” in terms of cellular supplementation. Addition of 10,000 SVF cells to 200 μ l of fat significantly improved fat graft retention. This improvement was significant not only when compared to unsupplemented fat grafts, but also when compared to all other supplemented groups with more cells. Furthermore, adding increased numbers of cells to fat grafts did not result in significantly improved fat graft retention; rather, supplementation with the maximum number of cells assessed in this study (10 million SVF cells) proved to negatively

impact volume retention. The lower volume retention observed in grafts receiving 10 million cells was accompanied by signs of lipodegeneration: decreased integrity, increased presence of cysts and vacuoles, and increased inflammation and fibrosis. These negative findings were compounded by the significantly decreased vascularity seen in this group.

Given the known pro-vasculogenic/angiogenic character of ASCs, the observation that a certain amount of SVF supplementation improves fat graft vascularity and volume retention is not surprising. *In vitro* studies have documented the ability of ASCs to promote neovascularization and angiogenesis via paracrine effects (i.e. release of VEGF) that positively influence vessel formation by both endothelial cells and endothelial progenitor cells (EPCs) (38–40). However, the fact that such studies commonly employ *ex vivo* co-culture models may shed light on our disparate findings that low numbers (10,000 SVF cell group) enhanced fat graft vascularity, while high numbers (10 million SVF cells) negatively impacted neovascularization. Unlike the controlled environment of a cell culture dish, the *in vivo* environment of a newly placed fat graft is relatively hypoxic and nutrient-poor. Thus, it is likely that there is a threshold level at which, instead of aiding and encouraging the formation of new vessels by EPCs and endothelial cells, added cells simply serve as a large group of competitors for resources.

Cell competition is known to be a driving force in determination of organ size at the embryonic level, as well as in mature organs such as the liver and bone marrow (e.g. hematopoietic stem cells) (41). Though much remains to be elucidated regarding the precise mechanisms by which intercellular competition contributes to an organ's final volume, it seems to occur when two actively-dividing cell populations come into contact and recognize differences between their metabolic and/or proliferation rates; the "weaker" cell population senses its disadvantage and either ceases proliferating or undergoes apoptosis, leaving the "stronger" cells to continue to grow and multiply (41, 42). Among stem cells, competition normally occurs as cells are constantly turned over within the niche; when two populations of stem cells are unequally matched, one may out-compete the other and grow to dominate the single niche, potentially leading to pathologic states such as malignancy (43). In CAL, one can imagine that the additional cells added to the fat graft may serve as a second population that, while they may possess beneficial effects on grafted adipocytes, may also simultaneously outcompete grafted fat cells along with resident ASCs for limited nutrients (44). This effect may be further complicated by the fact that, in the case of CAL performed with SVF (as in our study), a majority of the added cells are not ASCs, and thus may not have beneficial effects on graft take. While we assume the clinical translatability of SVF-CAL outweighs any potential advantages of ASC-CAL, further studies comparing these two approaches would be of value.

Whether supplemental through addition of SVF or native in the grafted fat, ASCs are thought to facilitate graft retention by either providing pro-angiogenic/vasculogenic cues or replenishing the rapidly dying pool of ischemic adipocytes (27, 44). However, recent studies from our laboratory have suggested a greater contribution of added ASCs to ultimate fat graft volume through new vessel formation than from direct contribution to formation of mature adipocytes. Single-cell transcriptional analysis of labeled ASCs extracted from CAL fat grafts demonstrated upregulation of VEGF and FGF2, while increases in markers of

adipogenic differentiation were not appreciated. Furthermore, long-term detection of labeled cells was not appreciated in fat grafts, suggesting only transient residence of added ASCs (37).

Interestingly, while our study has determined an optimum ratio of 50,000 SVF cells/mL for fat transfer, Kølle et al. found that 20 million *in vitro*-expanded ASCs per milliliter significantly enhanced large-volume (30mL) human fat grafts(24). Though our study fundamentally differs in that we utilized freshly harvested SVF cells and a murine model, given findings that volume retention rates differ between fat grafts of different sizes (45), the relationship between fat graft volume and the number of added cells needed for maximum take may not be linear. Further experiments are undoubtedly needed to evaluate the role of transplanted cells in grafts of varying volumes, though the feasibility of such studies are limited by the availability of animal models, and, in the case of clinical studies, cost-effective methods for accurate quantification of results.

Our findings are also particularly notable in light of the recent study by Li et al. that determined an optimum concentration of fat grafts supplemented with both ASCs and PRP (25). Li and colleagues found superior fat graft retention among grafts receiving PRP with 10^5 ASCs/ml, similar to our most effective supplemental cell to fat ratio of 50,000 SVF cells per ml. Important differences between the experiments performed by Li et al. and our study, however, include their use of a non-traditional method of fat processing, overnight storage of the fat prior to grafting, use of plated ASCs, and the lack of baseline volume determination. Additionally, while PRP has garnered clinical interest as a means to improve fat transfer (46) and is thus an interesting variable to assay, Li et al. omitted evaluation of the effects of ASC supplementation without PRP (and thrombin). Therefore, our findings are unique in that they provide an estimate of the number of SVF cells alone needed to enhance small-volume fat graft take in a murine model of CAL, while illustrating the potential drawbacks of over-supplementation.

Conclusions

CAL is a promising technique to enhance fat graft take, though clinical studies have yielded mixed reports of its efficacy (22, 24, 47, 48). Our data suggest that such differences may be due to suboptimal cell-to-tissue ratios, and that elucidation of the ideal ratios for given volumes of fat will allow for consistent clinical success of CAL. Furthermore, as SVF is highly heterogeneous, the optimal number of cells necessary for SVF-mediated fat graft enhancement may differ from that necessary for ASC-mediated enhancement. As such, it is important to continue to refine our knowledge of adipose tissue and stem cell biology in order to facilitate improvement of surgical outcomes. Our study has determined a potential starting point for the supplementation of small-volume fat grafts with SVF cells in order to obtain maximum volume retention. Conversely, we have also defined a concentration of cells that may be detrimental to fat graft survival. Thus, while these cells may have great promise as a cellular therapy in a variety of settings, we must continue to probe their complex roles as a stem cell population in order to make full use of their clinical potential.

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Figure 1. Schematic demonstrating experimental setup. Lipoaspirate was processed for grafting and for SVF isolation. SVF cells were then added back to prepared fat. Four different cell concentrations were assayed in a murine model of CAL, along with a control group that received fat alone.

Figure 2.

CAL volume retention. (a) CT images of SVF-supplemented fat grafts at 8 weeks post-grafting. Fat grafts shown in yellow. (b) Average fat graft volume retention with and without SVF supplementation over 8 weeks. Grafts receiving 10,000 cells had significantly higher volume retention (* $p < 0.05$, ** $p < 0.01$) than fat alone. (c) Average volume retention of fat grafts at 8 weeks. Grafts receiving 10,000 cells had significantly more volume (* $p < 0.05$) while grafts receiving 10 million cells had significantly less volume (* $p < 0.05$) than control group with fat alone.

Figure 3.

(a) Representative hematoxylin and eosin staining of fat graft sections at 8 weeks. (b) Fat grafts receiving 10 million cells had lower integrity (* $p < 0.05$), more inflammation (** $p < 0.01$), and more fibrosis, along with significantly increased presence of cysts and vacuoles (* $p < 0.05$). Fat grafts supplemented with 10,000 cells had the most integrity and the least inflammation and fibrosis.

Figure 4.

CD31 immunostaining. (a) Immunofluorescent staining for CD31 (red) with DAPI counterstain (blue) at 8 weeks. (b) Quantification of CD31 staining demonstrated grafts receiving 10,000 SVF cells had significantly higher vascularity (** $p < 0.001$) while grafts receiving 10 million cells had significantly lower vascularity (* $p < 0.05$) relative to the control group containing fat alone.