Improvement in autologous human fat transplant survival with SVF plus VEGF–PLA nano-sustained release microspheres

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Abstract
Early neovascularization is important for autologous fat transplant survival. SVF cells are ideal seed cells. Both vascular endothelial growth factor (VEGF) and SVF cells can promote neovascularization. However, the half-life (about 50 min) of VEGF is too short to sustain an adequate local concentration. We have investigated whether VEGF–polylactic acid (PLA) nano-sustained release microspheres plus SVF cells can improve neovascularization and survival of transplanted fat tissues. SVF cells were harvested and constructed VEGF–PLA nano-sustained release microspheres in vitro. Human fat tissues was mixed with SVF cells plus VEGF–PLA, SVF cells alone or Dulbecco’s modified Eagle’s medium as the control. These three mixtures were injected into random sites in 18 nude mice. Two months later, the transplants were weighed and examined histologically; and capillaries were counted to quantify neovascularization. Hematoxylin–eosin (HE) and anti-VEGF stains were applied to reveal cell infiltration. The mean wet weight of fat in the SVF plus VEGF–PLA, SVF alone, and control transplants were 0.18 ± 0.013 g, 0.16 ± 0.015 g, and 0.071 ± 0.12 g, respectively; the differences between groups were statistically significant. More vessels were present in the SVF plus VEGF–PLA transplants than in the other two types. Transplants mixed with SVF cells also had an acceptable density of capillaries. Histological analysis revealed that both the SVF plus VEGF–PLA and SVF alone transplants, but not the control transplants, were composed of adipose tissue, and had less fat necrosis and less fibrosis than control specimens. SVF plus VEGF–PLA transplants had significantly greater capillary density and VEGF expression than the other two transplant groups. Thus transplanted fat tissue survival and quality can be enhanced by the addition of VEGF–PLA nano-sustained release microspheres plus SVF cells.

Keywords: adipose-derived stem cells; adipose granule transplantation; nanosphere delivery system; stromal vascular fraction (SVF); vascularization; VEGF-PLA

Introduction
Correction of soft tissue defects by autologous fat grafting or injection was introduced by Neuber a century ago (Gurney, 1938). It is considered an ideal means for such repairs and is being increasingly accepted by clinicians and patients. The procedure is easy to perform and is associated with little donor site morbidity (Billings and May, 1989; Chajchir and Benzaquen, 1989), but its application is limited by graft absorption and necrosis-associated fibrosis (Chajchir et al., 1990). Although fat transplantation techniques have been refined and outcomes in many facilities have improved (Coleman and Saboeiro, 2007; Missana et al., 2007) in recent years, further improvement is needed. Adequate blood perfusion soon after transplantation seems to be critical to the fate of fat tissue grafts (Karacaglolu et al., 2005; Yamaguchi et al., 2005; Ogawa et al., 2007).

Adipose-derived stem cells (ADSCs) are commonly considered suitable seed cells. However, culturing ADSCs takes time and thus they are inconvenient for clinical application. Increasingly, SVF cells freshly isolated from adipose are being recognized. SVF cells from human adipose tissue have been described previously as including mesenchymal stem cells with multipotency that can differentiate into mesenchymal cells in vitro, including chondrocytes, osteoblasts, and myoblasts as well as adipocytes (Zuk et al., 2002). SVF cells can develop into a diversity of cell types, endothelial progenitor cells among them (Asahara...
et al., 1999), and when applied topically, they improve flap viability (Lu et al., 2008). Vascular endothelial growth factor (VEGF) and hepatocyte growth factor may strengthen this effect. Sheng et al. (2013) found that transplantation of SVF could shorten tissue expansion process through enhancing skin regeneration and secret pro-angiogenesis factor (such as EGF, VEGF, bFGF, etc.) which improve tissue expansion.

Neovascularization is regulated by VEGF under pathological as well as physiological conditions. VEGF promotes vascular permeability, endothelial cell proliferation and migration, and angiogenesis, prolongs the viability of vascular endothelial cells, and enhances blood vessel permeability. Hence, therapeutic manipulation of VEGF may improve ischemic tissue survival (Isenberg et al., 2007; Zheng et al., 2008). When applied topically or injected intravenously, VEGF enhances the revascularization of ischemic tissues such as skin flaps (Li et al., 2000; Zhang et al., 2001). There is positive feedback between VEGF and its receptors, in that stimulation of expression of VEGF and its receptors by endothelial cells increases the effects of VEGF. However, sustained local effective concentrations of growth factors are critical for mature blood vessel development, and the half-life (about 50 min) of VEGF is too short to achieve this (Huang et al., 2007). Feng et al. (2009) found that VEGF transfected ADSCs can extend survival of human autologous fat grafts, but adenoviral infection may cause harm to patients.

Sustained release technology is likely to be a solution to this problem. VEGF can be delivered within polylactic acid (PLA) microspheres that enable the cytokine to be released into the surroundings and exert its biological effects continuously. Moreover, PLA microspheres are non-toxic and are completely degraded in the body. This slow release technique also has great research value in the adipose tissue engineering field.

The aim of this study was to explore whether mixing SVF cells and VEGF–PLA nano-sustained release microspheres with fat tissues before transplantation enhanced neovascularization and increased fat graft survival. There has not been any report about these issues.

**Materials and methods**

**Source of human fat tissues**

Subcutaneous adipose tissue was harvested from one 30-year-old woman undergoing skin grafting. All of the fat samples obtained were collected as a (waste) byproduct of surgery. The individual in this manuscript gave written informed consent for the case details to be published. Ethical approval for this investigation was obtained from the Research Ethics Committee at Wenzhou University School of Medicine.

**Isolation and preparation of SVF cells**

SVF cells were isolated from the donor’s subcutaneous adipose tissue, which was minced into ~0.1-cm³ pieces and digested for 30 min in 0.125% collagenase (Sigma, USA) with intermittent shaking in a warm water bath (37°C) in a 50-cm³ centrifuge tube. The digestion process was halted by adding Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal bovine serum (FBS) and penicillin. The cell suspension was centrifuged for 5 min (1200 rpm/min, 25°C). Upon removal of the supernatant, the pellet containing the cellular fraction was re-suspended immediately in fresh DMEM with 10% FBS. The cell pellet was re-suspended and the cells were quantitated with the aide of trypan blue dye for visualization and plated in DMEM (1 × 10⁶ cells per 100 mm² tissue). The cells were then stored at 37°C and 5% CO₂ for the duration of the experiments.

**Construction of VEGF–PLA microspheres**

We constructed VEGF–PLA sustained release microspheres using in vitro ultrasonic emulsification. Briefly, VEGF in phosphate-buffered saline solution was mixed with an organic solution of poly (lactic acid) (PLA) with the ultrasonic dispersion method. We added polyvinyl alcohol (PVA), removed the organic solvent, applied ultrasonic emulsification, and then centrifuged the solution (1000 rpm). Smears of the resultant VEGF–PLA colloidal solution was stained with 3% phosphotungstic acid (PTA) at room temperature. After drying, we observed the encapsulation state under a transmission electron microscope and measured the microsphere diameters. We measured the optical density (OD) of each hole at 450 nm wavelength (Y-axis) using an MTT enzyme-linked immunometric meter, take the logarithm of VEGF concentrations as X-axis, and then drew a standard curve using Curve Exert1.3 software.

Drug loading and encapsulation rates were calculated according to the following formulas: Drug loading rate = Dose in the microspheres/Total weight of microspheres × 100%; Encapsulation rate = (Total dose in the system – Total dose in supernatant)/Total dose in the system × 100%. We placed the samples into Eppendorf test tubes, based on the calculated drug load, and subjected the tubes to low-speed oscillation in a constant-temperature water bath (37°C, 20 rpm/min). We measured VEGF levels 2 days later and every 2 days thereafter up to 24 days, calculated the cumulative release rate, and drew a best-fit curve for the cumulative release rate and time.

**Animal model and groups**

All procedures in this study were performed in accordance with the guidelines of Wenzhou Medical College Animal...
Care and Use Committee. The graft survival rates were 56 and 41% for the case and control groups, respectively. The capillary density values were 26.7 capillaries (per 0.2 mm$^2$) and 16.8 capillaries (per 0.2 mm$^2$) for the case and groups, respectively (see Yi et al., 2006). Using nQuery Advisor 6.0, we determined that an $N$ of 18 per group was suitable for our study.

Eighteen nude mice (15–18 g) were used as free fat transplantation models. Each mouse received a subcutaneous injection of fat tissue at three sites (0.4 mL/site); the fat grafts were mixed with 0.1 mL of $1 \times 10^6$ SVF cells plus VEGF–PLA nano-sustained release microspheres (group 1), 0.1 mL $1 \times 10^6$ SVF cells (group 2), or 0.1 mL DMEM (group 3). A total of 54 adipose grafts were transplanted; all three fat mixtures were administered to each mouse (one mixture per site). To control for possible inter-site regional blood supply differences, the sites were selected randomly. Ultimately, bleeding was not observed after any of the transplant procedures, indicating that there were no substantial musculocutaneous perforators, and further suggesting that the vascularity of the three sites was reasonably similar. Thus, all three fat preparation mixtures were injected into comparable locations across the 18 murine subjects.

**Weighing the transplants**

Two months after the injections, all animals were sacrificed. Each graft was excised and then weighed.

**Histopathology**

The graft specimens were fixed in formalin solution (10% v/v) and then prepared for slicing by being embedded in paraffin. Specimen sections taken from the central region of each biopsy were submerged in hematoxylin and eosin (HE) stain and labeled with anti-VEGF monoclonal antibody and then examined by light microscopy.

**Measurement of capillary density**

The expression of CD31, surface marker of neovascular endothelial cells, was detected by immunohistochemical staining. To quantitate neovascularization, capillaries were counted in multiple fields ($n = 20$, random selection methods were used to eliminate bias) within CD31-stained fat graft sections. Evaluation of 20 fields per section taken from the surviving fat tissue allowed us to determine capillary density, which is an index of revascularization. The capillaries were counted by two researchers who were blind to the specimen’s group designation at 400 × magnification. Capillary density data are reported as the mean number of capillaries ± standard deviation ($\bar{x} \pm s$).

**Statistical analysis**

The data are reported as means with standard deviations and subjected to statistical analysis in SPSS software (version 15.0; SPSS, Chicago, IL). Analysis of variance (ANOVA) was used to compare the means for the three fat tissue groups (randomized block design). Using Levene’s test, we tested whether the variance was sufficiently similar between the groups to meet an assumption of equal variance. If that assumption was found to be false, we applied Welch’s method of correcting the ANOVA $F$ statistic. If the overall test revealed significant differences between the means, then multiple pair-wise comparisons were conducted using either the least significant difference (if there was variance homogeneity) or applying the Games–Howell method (if there was not). $P$ values <0.05 were accepted as statistically significant in all cases.

**Results**

**Characterization of SVF cells**

Harvested SVF cells appeared round, translucent, and uniformly distributed under light microscopy. The cell surface was smooth (Figure 3b).

**VEGF–PLA microspheres**

In vitro, the surface of the microspheres was smooth, the sphere size was uniform (Figure 3c) with an average diameter of 27.56 ± 4.60 nm, the rate of encapsulation was 89.24 ± 1.24% and the drug loading rate was $(17.85 \times 10^{-3})\%$. There are quantitative relations between the logarithm of VEGF concentrations and OD450 (Figure 1). The cumulative release rate (%) and time (day) are being related in the standard curve (Figure 2). VEGF release from the microspheres was measured over a period of 24 days, over which the cumulative release rate was 80.49%.

![Figure 1 Standard curve showing the absorbance of VEGF concentration at OD450 (Y-axis) as a function of the log of VEGF concentration (X-axis). Note that there is a clear quantitative relationship between the log of VEGF concentration and absorbance.](image)
Injected tissue survival

No animals died during the 2-month posttransplantation observation period. At the conclusion of that period, the rats were executed and the fat grafts were weighed (Figures 3d–f). The mean wet weight of the fat grafts differed significantly between the SVF cells plus VEGF–PLA nano-sustained release microsphere group (group 1, 0.178 ± 0.013 g), the SVF cells group (group 2, 0.155 ± 0.015 g), and the DMEM control group (group 3, 0.071 ± 0.124 g) ($P < 0.05$ for all inter-group comparisons).

Histological evaluation of fat transplants

DMEM control transplants had extensive fibrosis and necrosis (group 3 in Figure 4a), whereas the SVF transplants had less fibrosis, infiltration of fat cells, and necrosis (group 2 in Figure 4b). The transplants mixed with SVF cells and VEGF–PLA nano-sustained release microspheres (group 1 in Figure 4c) were characterized by a predominance of surviving mature adipose tissue. The group 1 specimens had less fibrosis and fat necrosis than was observed in the other groups. The normal adipose histological structure was apparent in the transplants in all three groups. Levels of VEGF expression in group 1 (Figure 4f) were greater than that in the other two groups (group 2 in Figure 4e, group 3 in Figure 4d).

Capillary density

As shown in Table 1 and Figure 5, histological evaluation (20 fields per section from graft centers) revealed a significantly higher capillary density in the SVF plus VEGF–PLA group (Figure 6a) than in the other two groups. The transplants

![Figure 2](image-url) Gradual release of VEGF by microspheres over ~3 weeks. By 24 days, the cumulative release rate was 80.49%. X-axis (time, day), Y-axis (cumulative release rate, %).

![Figure 3](image-url) Microspheres and fat grafts. a: The fat particles after digestion of collagenase. b: SVF cell. Under microscopy, it showed round cells with smooth surfaces and translucent, distributed uniformly. Scale bar in subpart b indicates 100 μm. c: The VEGF–PLA nano-sustained release microspheres. The surface of the microspheres was smooth and the sphere size was uniform, with an average diameter of 27.56 ± 4.60 nm. Scale bar in subpart c indicates 100 nm. d,e: Fat grafts under the skin and with skin removed. f: The grafts from each group differed significantly in size from the grafts in the other two groups (mean wet weights reported in the Results Section).
with SVF cells alone (Figure 6b), which appeared to have adequate capillary density, had a higher density of capillaries than control transplants (Figure 6c), in which few capillaries were observed.

Discussion

Autologous fat transplantation is ideal for filling soft tissue and correcting contour defects, owing to its low cost, ease of harvesting, availability in large quantities, and autogenicity. Nevertheless, it commonly produces local calcifications that can appear suspicious in mammograms (Gradinger, 1987; Hartrampf and Bennett, 1987). Moreover, half the weight and volume of autologous fat transplants can be lost after transplantation (Peer, 1950; Chajchir et al., 1990). Although improvements in fat transplantation techniques have enabled clinically successful breast reconstruction (Coleman and Saboeiro, 2007; Missana et al., 2007), further advances in autologous fat transplantation are required. Revascularization of autologous fat transplants has been reported to occur after 48 h (Fawcett, 1948), probably due to its diffuse and highly metabolically active nature. This delay reduces fat cell survival, leading to degenerative changes that may result in transplant tissue absorption and the development of fatty cysts (Brownsey et al., 1984). Early and abundant neovascularization should improve survival of transplanted fat cells, and thereby improve transplant outcomes.

The process of angiogenesis—the formation of blood capillaries by endothelial cell sprouting from vessel walls (Brownsey et al., 1984) is regulated by various growth factors, including VEGF (Ogawa, 2006). VEGF is a major regulator of neovascularization under physiological and pathological conditions (Gurney, 1938), being a potent endothelial cell mitogen and also a differentiation signal for endothelial cell progenitors. It induces microvascular permeability, mitogenic effects on endothelial cells (Risau, 1997), endothelial cell proliferation and migration (Thomas, 1996), interstitial collagenase expression, and migration of macrophages (Senger et al., 1983). Nevertheless, VEGF's short half-life in vivo (~50 min) (Unemori et al., 1992) limits the capacity
for exogenous VEGF to produce an angiogenic effect. Furthermore, a high initial concentration can produce serious secondary effects, including improper vascularization of non-target sites or distant tumorigenesis, and the technique is expensive. Growth factors must be sustained at high local concentrations to support the development of mature blood vessels (Clauss et al., 1990). Hence, for therapeutic application, a delivery system is needed in which the VEGF supply is not only sustained, but also localized. PLA has good biocompatibility and is non-immunogenic since it has no peptide chain structure (Lazarous et al., 1996). The results of the present study indicate that VEGF–PLA can be used as a drug delivery system that provides a bioactively stable agent for weeks (Tan et al., 2011).

Recent developments in stem cell research have extended cell therapy approaches to promote transplant revascularization and support wound healing (Vega et al., 2012). Thus far, many cells, including mesenchymal stem cells (Khan et al., 2011), ADSCs (Kamihata et al., 2001), SVF cells and endothelial progenitor cells (Miranville et al., 2004), have been used to promote the revascularization of ischemic tissue.

ADSCs are ideal seed cells that multiply readily and are easily harvested. They are angiogenic and can differentiate into vascular endothelial cells in vitro. However, it takes 2–3 weeks to obtain sufficient ADSCs for transplantation, so this method is not suitable for clinical application. SVF do not need to be manipulated or expanded in culture and have the advantage of rapid extraction. Prior multicolor flow cytometry experiments revealed that samples of operatively aspirated adipose contained ADSCs, endothelial cells, and other cells following digestion of mature adipocytes and connective tissue. The harvested fat could be converted into

<table>
<thead>
<tr>
<th>Group</th>
<th>N</th>
<th>Mean no. vessels (±SEM)</th>
<th>Vs. group 2</th>
<th>Vs. group 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. SVF plus VEGF–PLA</td>
<td>20</td>
<td>14.75 ± 2.02</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>B. SVF</td>
<td>20</td>
<td>12.65 ± 1.69</td>
<td>–</td>
<td>0.001</td>
</tr>
<tr>
<td>C. DMEM (control)</td>
<td>20</td>
<td>7.25 ± 1.83</td>
<td>0.001</td>
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Figure 5 Histograms of the number of vessels in three groups. The number of vessels was greater in group 1 (transplants mixed with SVF cells plus VEGF–PLA nano-sustained release microspheres) than in the other two groups. Transplants mixed with SVF cells alone (group 2) had adequate capillary density. Capillaries were sparse in the control transplants. These differences were statistically significant.

Figure 6 The expression of CD31, surface marker of neovascular endothelial cells, was observed under microscope (CD31 monoclonal staining, 400×). The microvessel density was calculated. a: Transplants that were mixed with SVF cells plus VEGF–PLA nano-sustained release microspheres (group 1). b: The SVF-treated transplants (group 2). c: The DMEM control transplants (group 3). All transplants exhibited the histological structure of normal fat tissue.
SVF-rich fat (CAL) by adding SVF cells immediately upon their isolation, without manipulation or expansion in culture. Yoshimura et al.’s (Asahara and Kawamoto, 2004; Planat-Benard et al., 2004; Yoshimura et al., 2008) study shows that SVF have a broad clinical prospects. The amenability of SVF-rich fat to direct injection makes it potentially applicable to cosmetic breast augmentation and facial lipoatrophy correction. Jurgens et al.’s (Jurgens et al., 2013) study shows safety and feasibility of a one-step surgical procedure for osteochondral cartilage regeneration using freshly isolated adipose stromal cells. They also found that freshly isolated adipose stromal cells are at least equal to cultured ASCs in this procedure. When employing the CAL strategy, SVF cells are more suitable for transplantation than ADSCs. Meanwhile, SVF cells are used for treatment of the donors themselves, so SVF cell therapy is both legal and safe.

We chose to do the present experiments in nude mice because of their immunodeficiency, with the aim of minimizing immune rejection of the grafts. Additionally, all grafts were from the same donor to reduce the sampling error. In the present study, our histological observations revealed that fat transplants that had been combined with SVF plus VEGF–PLA exhibited less fat necrosis, less fibrosis, and more mature adipocytes than SVF alone and DMEM control transplants. These findings suggest that SVF plus VEGF–PLA treatment could improve autologous fat transplantation efficiency. We observed the expression of CD31 which is the surface maker of neovascular endothelial cells and calculated the microvessel density. We found the mean weight and capillary density of fat transplants in the SVF plus VEGF–PLA group were significantly greater than those of the SVF transplants, which were significantly higher than those of the DMEM control transplants. Thus, we deduced that SVF cells can improve free fat transplant survival and that VEGF–PLA can strengthen this benefit.

The mechanism by which SVF cells plus VEGF–PLA improved fat transplant survival is not known. However, our data are consistent with several possibilities. SVF cells, which include ADSCs, endothelial cells, etc., not only secrete cytokines that stimulate adipocyte survival and neovascularization, but also differentiate into vascular endothelial cells and adipocyte cells. Thus, the differentiation of ADSCs into vascular endothelial cells may promote vasculogenesis, thereby supporting transplant survival. Additionally, some stem cells among the SVF cells could differentiate into mature fat cells that contribute to the constitution of the transplant; such differentiation could compensate for early losses of adipocytes in the graft. Lastly, hypoxic conditions experienced by untransduced stem cells early after transplantation could induce the cells to express angiogenic factors, such as VEGF, which promote rapid neovascularization and thereby enhance adipocyte survival. However, secreted VEGF is insufficient to exert such an effect. VEGF–PLA could provide a prolonged effective local concentration of VEGF that could promote the differentiation of stem cells and vasculogenesis.

In conclusion, our results indicate that SVF cells plus VEGF–PLA provided a sustained effective local VEGF concentration and produced greater capillary density and fat weight than SVF cells alone. These findings strongly support the notion that VEGF–PLA plus SVF cells can significantly improve the neovascularization and viability of autologously transplanted fat transplants. Our study demonstrates that these two therapeutic approaches may be more efficacious in combination. It also demonstrates the preclinical safety and feasibility of a one-step surgical procedure using SVF cells plus VEGF–PLA. Larger studies with longer follow-up are required to substantiate these findings.

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References


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