Comparison of Three Different Fat Graft Preparation Methods: Gravity Separation, Centrifugation, and Simultaneous Washing with Filtration in a Closed System

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Background: Successful long-term volume retention of an autologous fat graft is problematic. The presence of contaminating cells, tumescent fluid, and free lipid in the graft contributes to disparate outcomes. Better preparation methods for the fat graft before transplantation may significantly improve results.
Methods: Subcutaneous fat from 22 donors was divided and processed using various graft preparation methods: (1) no manipulation control, (2) gravity separation, (3) Coleman centrifugation, and (4) simultaneous washing with filtration using a commercially available system (Puregraft; Cytori Therapeutics, Inc., San Diego, Calif.). Fat grafts from various preparation methods were examined for free lipid, aqueous liquid, viable tissue, and blood cell content. Adipose tissue viability was determined by measuring glycerol release after agonist induction of lipolysis.
Results: All test graft preparation methods exhibited significantly less aqueous fluid and blood cell content compared with the control. Grafts prepared by washing with filtration exhibited significantly reduced blood cell and free lipid content, with significantly greater adipose tissue viability than other methods.
Conclusion: Washing with filtration within a closed system produces a fat graft with higher tissue viability and lower presence of contaminants compared with grafts prepared by alternate methods. (*Plast. Reconstr. Surg.* 131: 873, 2013.)

Autologous adipose tissue has been used for soft-tissue repair and filling applications for over a hundred years. Unfortunately, tissue volume loss ranging from 25 to 90 percent often leads to unpredictable outcomes.[1] One reason for unpredictable results is the application of various techniques during tissue harvest, processing, and reimplantation. Many investigators have emphasized the importance of removing the nonviable, proinflammatory components of lipoaspirate, including oil, blood cells, damaged tissue, and debris, before reimplantation.[2,3] Removal of these components is typically achieved using techniques such as centrifugation,[4] washing with filtration,[5,6] or simple unit gravity sedimentation and decantation.[5,7]

In the present study, a side-by-side, in vitro comparison was conducted that looked at commonly applied graft preparation methods: gravity separation, centrifugation, and tissue washing with filtration using a commercially available product. The composition of grafts prepared by each method was evaluated to determine the processing method that might best promote graft survival by removing contaminants and preserving viable adipose tissue.

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MATERIALS AND METHODS

Fat Source and Harvesting Methods

Subcutaneous adipose tissue was harvested from 22 healthy female donors (age range, 24 to 64 years; mean age, 45 ± 12 years). Tissue was obtained from the abdomen, hips, and flanks.

Disclosure: *Dr. Zhu, Mr. Hicok and Mr. Shanahan, and Drs. Arm and Fraser are shareholders and employees of Cytori Therapeutics, Inc. Dr. Cohen is a shareholder and paid consultant for Cytori Therapeutics. Dr. Strem is a former employee of Cytori Therapeutics. Mr. Yu is an employee of Cytori Therapeutics. Cytori Therapeutics provided funding and technical and editorial support for this study.*

Informed consent was obtained using an independent Institutional Review Board–approved protocol (Independent Review Consulting, San Anselmo, Calif.).

Fat Graft Preparation Techniques

Lipoaspirate was mixed to create a homogeneous suspension and then divided into four portions, one for each preparation method: control (30 ml, no preparation), gravity separation (60 ml in a single Toomey syringe), centrifugation (60 to 100 ml in 10-ml syringes), and simultaneous tissue washing with filtration (200 ml or 1000 ml using the Puregraft product; Cytori Therapeutics, Inc., San Diego, Calif.). Control samples were analyzed without subsequent manipulation. Gravity-separated samples were maintained at room temperature for 20 minutes or until fluid and adipose tissue layers were separated. The bottom aqueous layer was drained away from graft tissue before further analysis. Centrifugation samples were placed in an IEC fixed-angle rotor centrifuge (International Equipment Company, Needham Heights, Mass.) and centrifuged at 3000 rpm (1200 *g*) for 3 minutes. Three distinct layers of free lipid (oil), adipose tissue (the graft), aqueous infranatant, and a pellet composed of blood cells and debris were observed. The free lipid and aqueous layers were discarded, as described by Coleman.[11] Graft samples in the simultaneous washing with filtration arm of the study were processed using the Puregraft system according to the manufacturer's instructions. Fat was washed twice with lactated Ringer's solution at the ratio of one volume of wash to two volumes of tissue. Drain time after each wash was approximately 3 minutes.

Microscopic Evaluation

Adipose tissue samples collected from each test group were prepared by spreading the tissue onto microscope slides. Samples were examined for the presence of free lipid and blood cells. Representative images were captured at 100× magnification using a Spot camera and Spot digital image capture software (Spot Imaging Solutions, a division of Diagnostic Instruments, Inc., Sterling Heights, Mich.).

Aqueous Liquid and Free Lipid Content of the Grafts

Triplicate 10-ml samples from each graft processing method were centrifuged at 400 *g* for 5 minutes at room temperature. After centrifugation, different density components were measured from top to bottom: free lipid layer, adipose tissue, an aqueous liquid layer composed of residual tumescent/wetting solution or wash solution, and a cell pellet composed of blood cells, loosely adherent cells, microvasculature, and extracellular matrix fragments. Volumetric measurements of the free lipid layer and aqueous layer were recorded and reported as relative content of total precentrifugation volume.

Blood and Loosely Adherent Cell Content of the Grafts

To quantify cells, cellular pellets from each separated graft sample were retrieved and resuspended using a Beckman Coulter NucleoCounter (model AC.T10; Beckman Coulter, Inc., Brea, Calif.). The number of red blood cells and white blood cells per gram of graft material was then calculated. Results from each lipoaspirate sample were averaged and expressed as a percentage of the control value.

Lipolysis Assay

A free glycerol determination kit and glycerol standard solution (both from Sigma/Aldrich, St. Louis, Mo.) were used to measure free glycerol released from adipose graft samples in triplicate. Aliquots (300 mg) of adipose tissue were placed into a 24-well cell culture plate containing assay buffer (Dulbecco's modified Eagle medium/F-12 medium; CellGro, Herndon, Va.) at a final concentration of 4% human serum albumin (JRH Biosciences, Lenexa, Kans.) and incubated for 3.5 hours at 37°C. Glycerol release for each sample preparation was determined after agonistic induction using 1 μM isoproterenol hydrochloride (Sigma, St. Louis, Mo.). Lipolysis was determined spectrophotometrically at a wavelength of 540 nm. A standard curve of adipocyte viability was obtained by mixing various ratios of viable tissue with tissue that had been rendered nonviable (heated to 95°C for 20 minutes), to demonstrate linearity of glycerol release as a function of viable adipocyte content.

Determination of Angiogenic Growth Factors in Fat Grafts

Fat grafts prepared by centrifugation and using Puregraft were mixed with equal volumes of two times lysis buffer containing proteinase inhibitor (RayBiotech, Norcross, Ga.). The samples were sonicated on ice and then centrifuged at 12,000 rpm for 15 minutes at 4°C. Protein concentrations of the samples were measured using the bicinchoninic acid protein assay. Cytokines

were measured with the RayBio Human Cytokine Antibody Array II Kit (RayBiotech), according to the manufacturer's instructions. Array images were quantified and final protein concentrations were normalized to total protein. The factors evaluated in this study are listed in Table 1.

Statistical Analysis

All data are reported as means ± SEM. Specifically, we used a mixed-effects linear model with preparation as a fixed effect and subject as a random effect to compare the various graft preparation techniques in terms of adipose tissue lipolysis activity, aqueous fluid content, free lipid content, and blood cell content. A covariance structure with unequal variances across the preparation methods was assumed. Histograms of the residuals and q-q plots were examined for violations of the normality assumption. Tukey's honestly significant difference method was used to correct for the multiple comparisons across the graft preparation techniques; Bonferroni's adjustment was used to correct for the multiple hypothesis tests for the five types of adipose tissue content. Overall statistical significance was defined as $p < 0.05$; using Bonferroni's multiple testing correction for the five characteristics considered (lipolysis, fluid content, lipid content, red blood cells, and white blood cells), individual p values were compared with 0.01.

RESULTS

Graft Characterization

Figure 1, *above, left*, shows the visual appearance of grafts prepared using various techniques.

Table 1. Factors Evaluated in Study Samples

Abbreviation	Full Name
IGF-1	Insulin-like growth factor 1
VEGF	Vascular endothelial growth factor
IFN-γ	Interferon-gamma
Thrombopoietin	Thrombopoietin
VEGF-D	Vascular endothelial growth factor D
IL-8	Interleukin 8
IL-6	Interleukin 6
PIGF	Placenta growth factor
TGF-β1	Transforming growth factor beta 1
ENA-78	Epithelial neutrophil-activating protein 78
MCP-1	Monocyte chemotactic protein 1
PDGF-BB	Platelet-derived growth factor BB
GRO	Growth-related oncogene
EGF	Epidermal growth factor
TIMP-1	Tissue inhibitor of metalloproteinase 1
Leptin	Leptin
TIMP-2	Tissue inhibitor of metalloproteinase 2
RANTES	Regulated upon activation, normal T cell–expressed and secreted
bFGF	Basic fibroblast growth factor
Angiogenin	Angiogenin

Fat grafts prepared by washing with filtration were obviously less red, suggesting lower red blood cell content than grafts prepared using the other methods. Microscopic evaluation (Fig. 1, *center* and *below*) also indicated that grafts prepared by washing with filtration with the Puregraft system exhibited lower red blood cell content as well as the reduced presence of microscopic droplets of free lipid compared with grafts prepared by either unit gravity sedimentation or centrifugation. This apparent difference was confirmed following centrifugation of the grafts to separate them into their constituent parts (Fig. 1, *above, right*).

Quantitation of these data for all donors showed that grafts prepared by gravity sedimentation had the highest relative aqueous content (24.5 ± 1.5 percent), whereas graft prepared by centrifugation had the lowest aqueous content (4.6 ± 0.4 percent). Grafts prepared using the Puregraft system had intermediate aqueous liquid levels (8.5 ± 0.7 percent for Puregraft 250 system and 7.3 ± 1.2 percent for Puregraft 850 system); these levels were significantly lower than those for control and gravity grafts (all Tukey-adjusted p values < 0.01) (Fig. 2, *above, left*). Extracellular lipid content within the grafts is shown in Figure 2, *above, right*. Grafts prepared by centrifugation had the highest lipid content (12.6 ± 1.6 percent), while those prepared with the Puregraft system had the lowest (0.48 ± 0.07 percent for Puregraft 250, and 0.11 ± 0.01 percent for Puregraft 850). The level of free lipid in grafts prepared by washing with filtration was statistically significantly lower than that in the control grafts, gravity grafts, and grafts prepared by centrifugation (all Tukey-adjusted p values < 0.01). Analysis of the cell pellet showed that gravity sedimentation and centrifugation techniques removed approximately 50 percent of the red blood cells and 60 to 70 percent of the white blood cells present in the original aspirate (Fig. 2, *below*). In contrast, washing with filtration using both the Puregraft 250 and the Puregraft 850 removed more than 95 percent of both blood cell types. This difference was statistically significant (all Tukey-adjusted p values < 0.01) with respect to both control grafts and grafts prepared by centrifugation. Given the poor quality of grafts prepared by gravity sedimentation alone (as determined by fluid content and the presence of cellular and lipid contaminants), subsequent studies looking more closely at the biologic properties of the graft focused only on grafts prepared by centrifugation or washing with filtration.

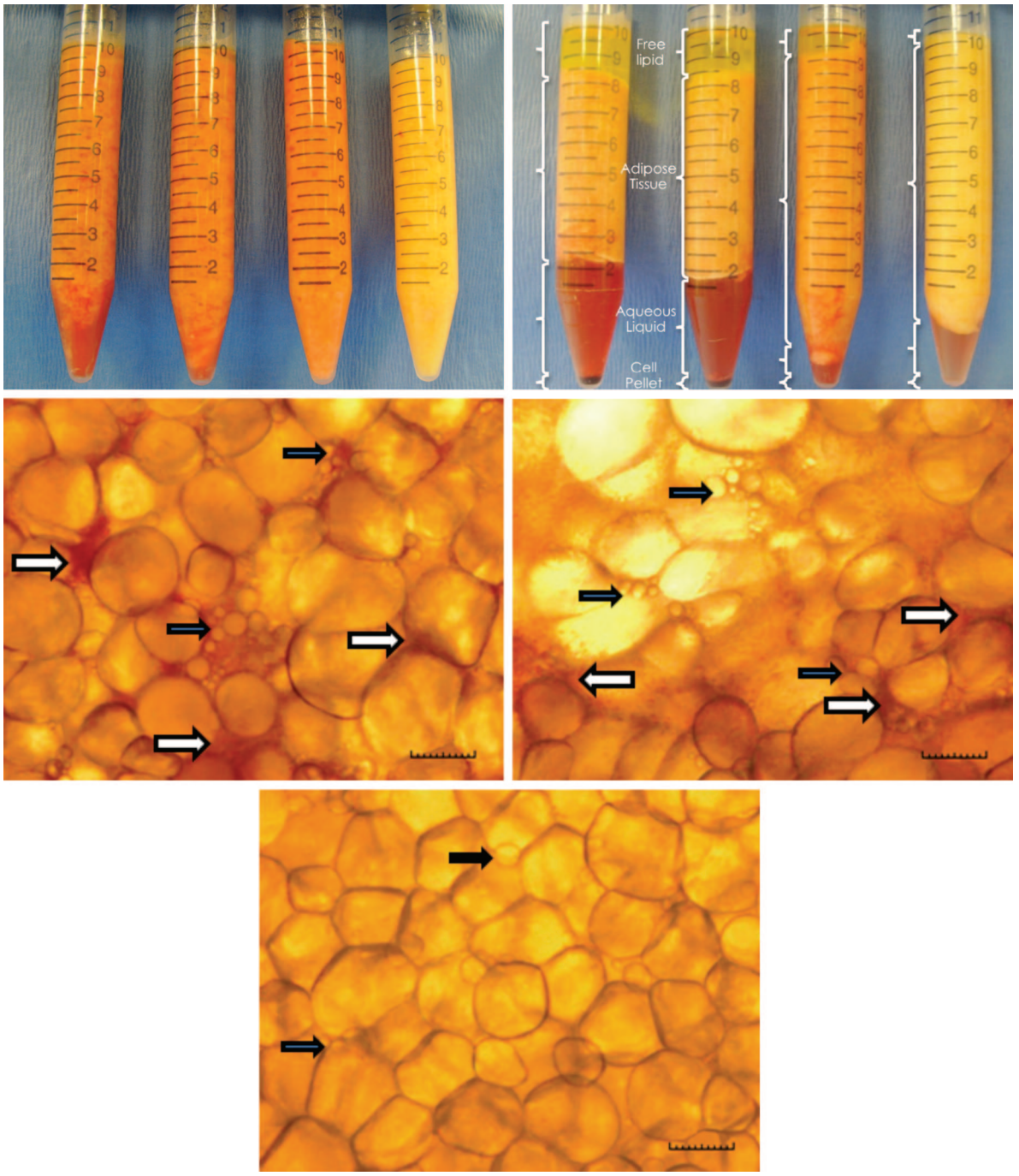

Fig. 1. Visual and microscopic appearance of fat grafts prepared by different methods. (*Above, left*) Representative samples after each graft preparation process (from *left* to *right*): control (unprocessed lipoaspirate), gravity separation, centrifugation, and washing with filtration (Puregraft). Microscopic evaluation of fat graft tissue processed with unit gravity sedimentation (*center, left*), centrifugation (*center, right*), and washing with filtration (*below*). *White arrows* indicate concentrated regions of red blood cells. *Black arrows* identify droplets of free lipid. (*Above, right*) Representative samples after centrifugation was used to separate the grafts into their four phase components [free (extracellular) lipid, adipose tissue, aqueous fluid, and cell pellet].

Adipose Tissue Viability

Glycerol release in response to adrenergic stimulation was linearly correlated with the relative content of viable tissue in the preparation (Fig. 3) ($r = 0.994$; $n = 5$). Using this assay, adipose tissue viability was found to be significantly higher for tissue processed with the Puregraft system (for both Puregraft 250 and Puregraft 850) as com-

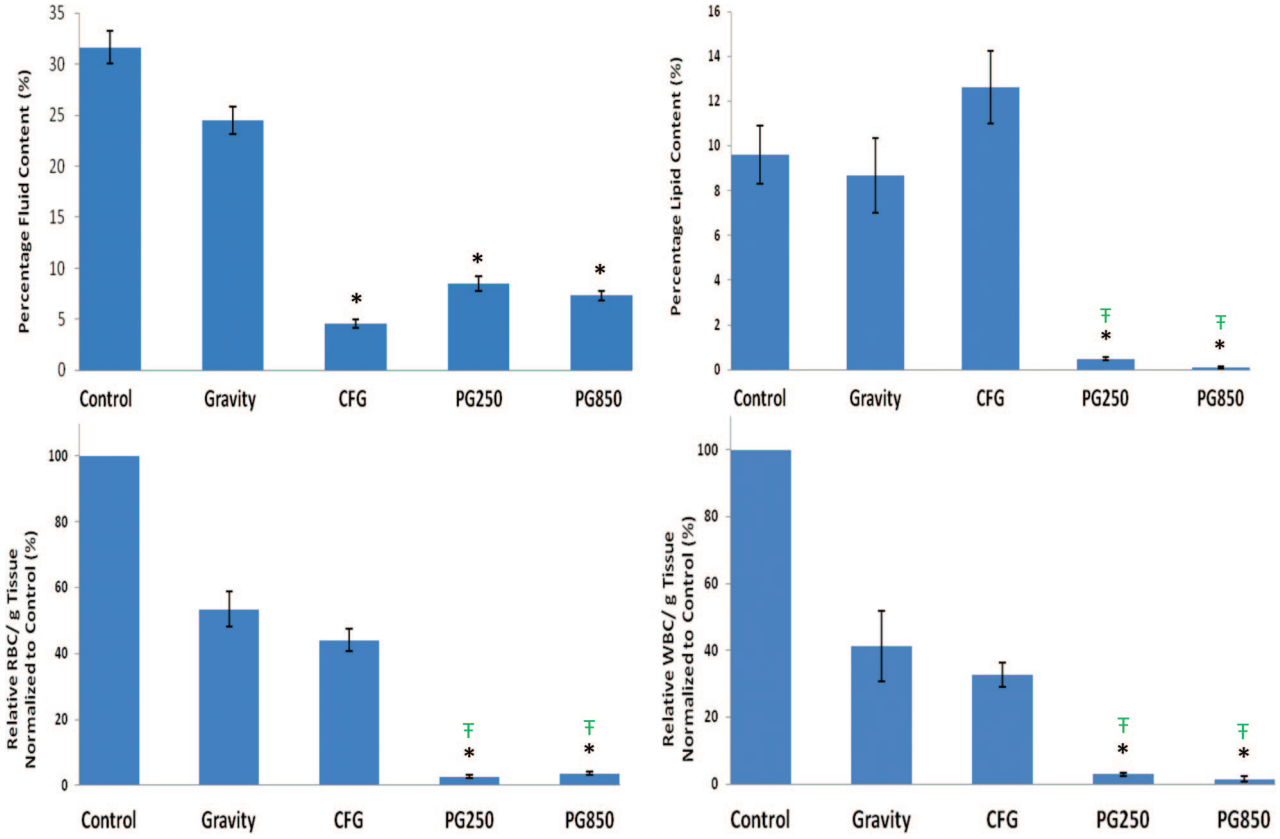

Fig. 2. Composition of grafts after separation into components by centrifugation: (*above, left*) aqueous liquid, (*above, right*) free (extracellular) lipid, (*below, left*) red blood cells (*RBC*), and (*below, right*) white blood cells (*WBC*). Data are presented as mean ± SEM ($n = 22$). *$p < 0.01$ when compared with control. $p < 0.01$ when compared with centrifugation graft preparation method. *CFG*, grafts prepared by centrifugation; *PG250*, grafts prepared with the Puregraft 250 System; *PG850*, grafts prepared with the Puregraft 850 System.

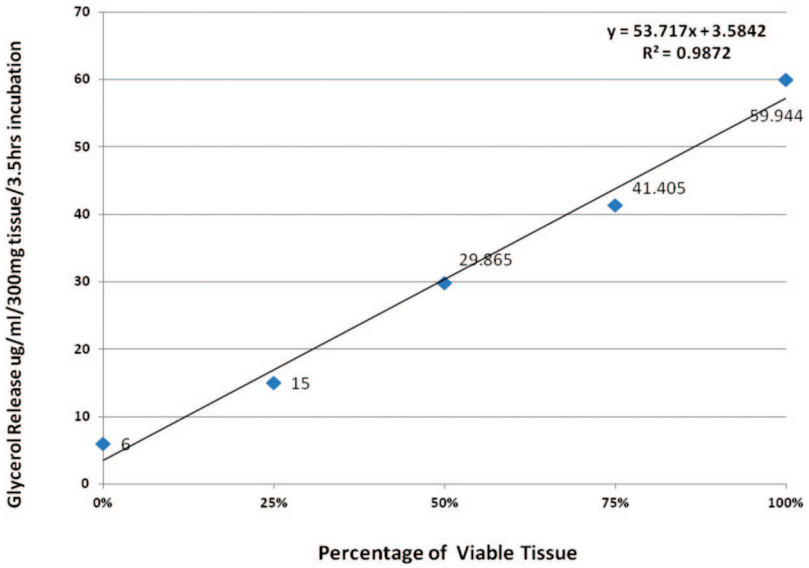

Fig. 3. Adipose tissue viability after graft processing was determined by correlating agonist-induced glycerol release to a validated correlation curve of glycerol release by known quantities of viable adipose.

pared with control (nonprocessed tissue) or grafts prepared by centrifugation (Fig. 4) (all Tukey-adjusted p values < 0.01).

Growth Factor Content

The results showed no significant difference in growth factor content (Table 1) between grafts prepared by centrifugation and those prepared by washing with filtration (Fig. 5) (all comparisons, $p > 0.05$, paired t test).

DISCUSSION

Autologous fat graft survival and long-term retention are influenced by the manner in which the tissue is harvested, processed, and delivered back to the patient.[2,8] This study focused on the processing component of fat grafting. The most common means for preparing grafts include centrifugation, washing with filtration, and unit gravity sedimentation and decantation.[7] These steps are applied to reduce undesirable components, such as blood, debris, and free lipid, and ruptured adipocytes, while retaining viable adipose tissue.[2,9] As with any surgical technique, control and standardization of technical variables lead to greater predictability of outcomes. In this study, different processing methods were compared head to head using the same tissue in an effort to evaluate the impact of processing on the composition of the graft.

While the aqueous fluid fraction of a fat graft aids in distribution during delivery, it contributes nothing to graft retention, as the fluid is rapidly resorbed by the body following implantation. Surgeons routinely compensate for this phenomenon by overfilling the graft site, although this is not always feasible due to volume constraints at the site of implantation. Our data showed that centrifugation and processing by washing with filtration using the Puregraft system were the most effective means of reducing the amount of aqueous liquid in the graft (Figs. 1 and 2, *above, left*). This is consistent with data from Kurita et al.,[10] who showed that centrifugation condenses the tissue, thereby reducing aqueous liquid content.

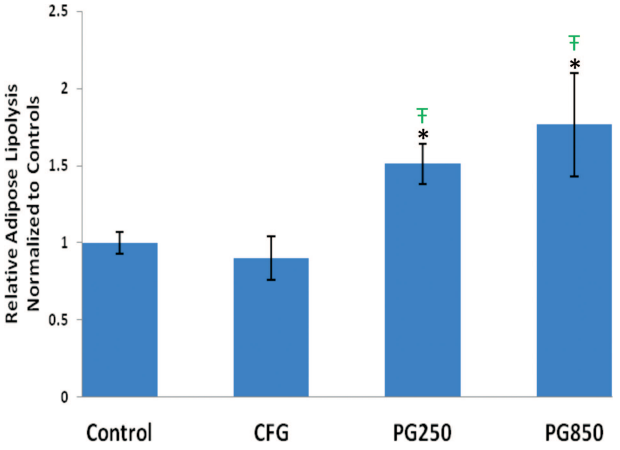

Fig. 4. Relative lipolysis activity of grafts prepared by centrifugation or closed washing systems. Adipose tissue metabolic activity was significantly higher after adipose tissue washing using the Puregraft systems compared with centrifugation ($p < 0.01$).

Removal of contaminants is another important factor in graft retention. Extracellular free lipid released from ruptured adipocytes can induce an inflammatory response. This material is scavenged by tissue phagocytes or walled off into lipid cysts by inflammatory cells in a foreign body response. To avoid these problems, lipid is routinely removed from grafts prepared by centrifugation.[11] However, microscopic evaluation of grafts prepared by gravity sedimentation or centrifugation reveals significant retention of lipid within the graft (Fig. 1, *center* and *below*). Indeed, our data show that extracellular lipid accounts for approximately 12 percent of the total graft volume in grafts prepared by centrifugation and approximately 8 percent of grafts prepared by gravity sedimentation (Fig. 2, *above, right*). By contrast, retention of this material is reduced to less than 1 percent in grafts prepared by washing with filtration. Similarly, once transplanted, extravasated blood cells are cleared by an inflammatory process that can lead to collateral damage and loss of graft volume. The data presented in this study show that centrifugation and gravity sedimentation remove approximately 50 to 60 percent of red and white blood cells (Fig. 2, *below*). This finding is similar to those of Rohrich et al.[12] and Kurita et al.[10] In comparison, washing with filtration removes more than 95 percent of both blood cell types. The ability of washing with filtration to remove both cellular and lipid contaminants with significantly greater efficiency is not surprising, given that centrifugation applies a single-stage separation whereas washing and filtration within the closed system of the Puregraft process allows two washing steps with a substantially larger volume of wash fluid within the same time period.

It might be argued that in addition to removing contaminants, the washing process might also wash out beneficial agents, such as growth factors. Our data (Fig. 5) demonstrate that this is not the case. That is, growth factor content was similar for all graft preparation methods. This is likely due to the fact that such factors are water-soluble and will, therefore, be extensively diluted by the tumescent

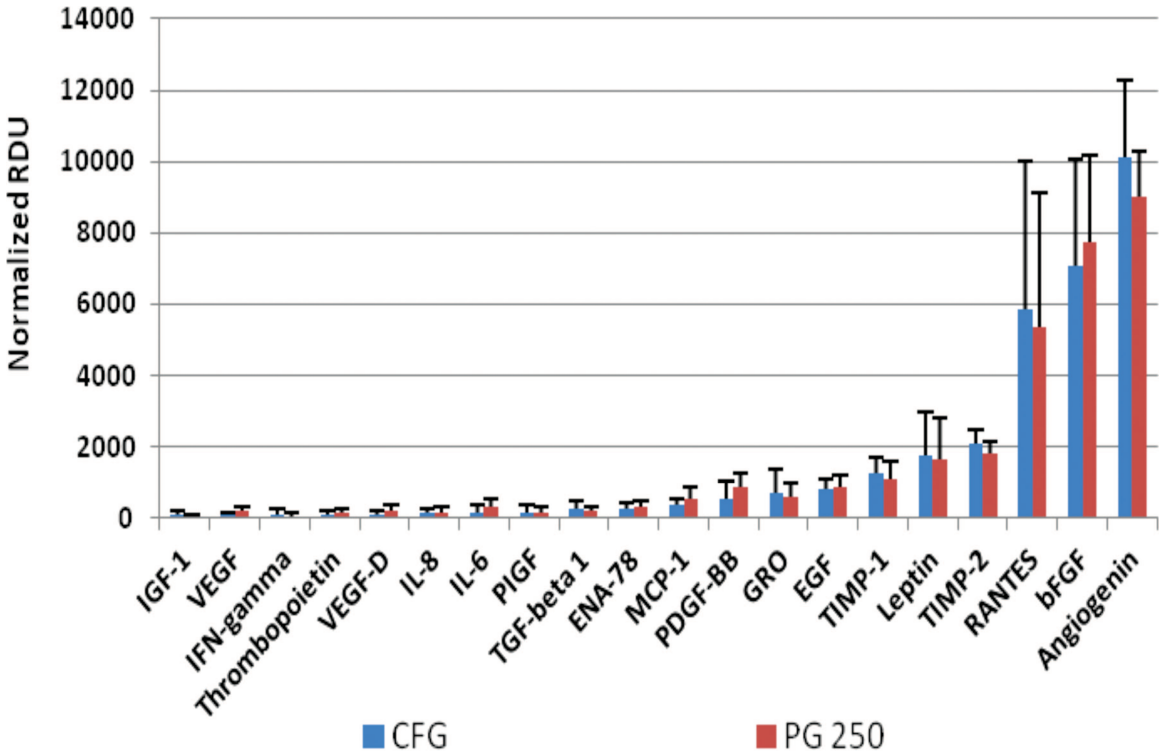

Fig. 5. Growth factor retention within the graft: normalized relative density unit (RDU) of growth factors expression profile ($n = 5$). Grafts prepared by centrifugation and washing with filtration were used to extract proteins for human cytokine determination. There was no significant or consistent difference in growth factor content in these grafts across a broad range of factors.

fluid added before lipoplasty. Furthermore, the very short biologic half-life of these factors[13–15] means that any release by cells during processing is irrelevant when compared with intracellular stores and the ongoing biosynthesis needed to sustain growth factor expression over the days and weeks necessary for graft revascularization after transplantation.[16]

Analysis of hormone-stimulated lipolysis showed significantly greater tissue viability in grafts processed by washing and filtration within the Puregraft system than in grafts prepared by gravity sedimentation or by centrifugation (Fig. 4). Given that the starting tissue was identical for all processing methods, these data suggest either that washing with filtration selectively enriches for viable tissue (perhaps by allowing damaged tissue fragments to pass through the filter) or that centrifugation damages the tissue. The latter interpretation is consistent with data from Smith et al.[17] and Xie et al.,[18] who showed reduced viability of centrifuged adipose tissue. However, in vivo studies in both humans[6] and small animals[17,19] have found no difference in graft retention as a result of centrifugation. It is possible that this may be due to the relative insensitivity of in vivo assays.

CONCLUSIONS

Graft tissue prepared by washing with filtration in a closed system exhibits significantly lower contamination by elements associated with a negative impact on graft retention (excess aqueous fluid, oil, and blood cells), while retaining viable functional adipose tissue. Initial clinical experience (S. R. Cohen, unpublished observations) indicates that washing and filtration using the Puregraft system can be successfully integrated into small- or large-volume fat transfer. Further clinical studies will be needed to determine whether these higher-quality grafts result in improved graft retention.

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