Numerical Measurement of Viable and Nonviable Adipocytes and Other Cellular Components in Aspirated Fat Tissue

Hirotaka Suga, M.D.
Daisuke Matsumoto, M.D.
Keita Inoue, M.D.
Tomokuni Shigeura, M.S.
Hitomi Eto, M.D.
Noriyuki Aoi, M.D.
Harunosuke Kato, M.D.
Hiroaki Abe, M.D.
Kotaro Yoshimura, M.D.

Tokyo and Yokohama, Japan

Background: A reliable method with which to assay viability and number of adipocytes and other cellular components in adipose tissue remains to be established.
Methods: The authors assessed cell viability and number obtained from 1 g of suctioned adipose tissue and respective layers (the top, middle, and bottom layers) before and after digestion and centrifugation, using cell staining with Hoechst 33342 and propidium iodide and the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2*H*-tetrazoliumhydroxide (XTT) and glycerol-3-phosphate dehydrogenase assays ($n = 10$). The correlation between the number of prepared cells (adipocytes, adipose stromal cells, and white blood cells) and the resulting values from the XTT and glycerol-3-phosphate dehydrogenase assays was also examined ($n = 5$). The cell composition of the stromal vascular fraction isolated from the same adipose tissue was determined by multicolor flow cytometry ($n = 5$).
Results: Hoechst 33342 and propidium iodide staining allowed distinguishing of viable adipocytes from lipid droplets, dead adipocytes, and cells other than adipocytes. The authors obtained 6.9×10^5 nonruptured adipocytes from 1 g of suctioned adipose tissue; 30 percent of the original adipocytes appeared to have been ruptured. Both the XTT and glycerol-3-phosphate dehydrogenase assays provided good correlations between the number of viable adipocytes and resulting values, but only the glycerol-3-phosphate dehydrogenase assay was strictly specific for adipocytes. The ratio of adipose stromal cells to adipocytes was found to be much larger than previously described.
Conclusion: Single use or a combination of the viability assays used in this study can appropriately determine the number of adipocytes and other cells, although it remains difficult to assess original cells directly without tissue dissociation. (*Plast. Reconstr. Surg.* 122: 103, 2008.)

Adipose tissue has been widely studied in basic research, especially in relation to obesity and metabolic diseases such as diabetes.[1] In the clinical setting, autologous fat grafting has long been an effective technique for soft-tissue augmentation. Furthermore, multipotent stem cells have been identified in adipose tissue, resulting in a new field in adipose biology.[2–7] Despite the intense interest in adipose tissue, however, its cellular composition—either excised or aspirated—remains unknown. Although cell counting with cell staining,[8–13] colorimetric assays—namely, the 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenyl-amino)carbonyl]-2*H*-tetrazoliumhydroxide (XTT) and/or 3-(4,5-2-yl)-2,5-ditetrazolium bromide (MTT) assays[9,14–17]—and the glycerol-3-phosphate dehydrogenase assay[9,10,18] have been used in previous studies to analyze cell number and viability in adipose tissue, a reliable method has not yet been established. Specifically, the number of mature adipocytes is difficult to

From the Department of Plastic Surgery, University of Tokyo School of Medicine, and the Division of Research and Development, Biomaster, Inc.
Received for publication July 20, 2007; accepted December 14, 2007.

DOI: 10.1097/PRS.0b013e31817742ed

Disclosure: *The authors do not have any commercial associations or financial disclosures that might pose or create a conflict of interest with information presented in this article.*

measure, mainly because of their fragility and the presence of adjacent progenitors.

Trypan blue staining is a well-established procedure for detecting viable cells. In adipocytes, however, this technique is difficult because of the scant cytoplasm of mature adipocytes.[12] Other staining methods should be used to distinguish viable adipocytes, and differentiating adipocytes and pure lipid droplets is also necessary.

A colorimetric assay uses tetrazolium salt (XTT and/or MTT), which can be reduced by active mitochondrial dehydrogenase in viable cells, yielding a highly colored formazan product.[19] This assay provides a good correlation between the number of viable cells and the resulting formazan absorbance values, but it is not specific for adipocytes. In addition, because adipose tissue contains not only adipocytes but also blood-derived cells, endothelial cells, and adipose-derived stem/stromal cells, these other cells could influence the total absorbance values.

In the glycerol-3-phosphate dehydrogenase assay, glycerol-3-phosphate dehydrogenase activity is determined by measuring the decrease in the reduced form of nicotinamide adenine dinucleotide concentration.[18] This assay has the advantage of adipocyte specificity, but extracellular glycerol-3-phosphate dehydrogenase from damaged adipocytes is measured together with intracellular glycerol-3-phosphate dehydrogenase from viable adipocytes, affecting the accuracy of viability analysis. Furthermore, a correlation between the number of viable adipocytes and the resulting values of glycerol-3-phosphate dehydrogenase activity has not been reported.

Thus, how many adipocytes and other cellular components exist in the adipose tissue remains unclear.[8,10,13] In this study, to seek a reliable method for evaluating adipose tissue, we assessed the viability and number of cellular components obtained before and after collagenase digestion of suctioned adipose tissue using cell staining, the XTT assay, and the glycerol-3-phosphate dehydrogenase assay.

MATERIALS AND METHODS

Tissue Sampling and Processing

After obtaining informed consent using an institutional review board–approved protocol, we acquired adipose tissue from 10 healthy female donors undergoing liposuction of the abdomen or thighs. We used a cannula with a 2.5-mm inner diameter and a conventional liposuction machine. From each donor, five sample tubes were prepared. We carefully collected the most homogeneous samples possible, making an effort not to include debris or clumps. The sample was divided by weighing 1 g for each specimen. One sample of the five was directly subjected to the XTT assay, and another sample was directly subjected to the glycerol-3-phosphate dehydrogenase assay. The other three samples were processed by digestion with collagenase and centrifugation; each sample was mixed with 2 ml of 0.075% collagenase (Wako Pure Chemical Industries, Osaka, Japan) in phosphate-buffered saline and incubated at 37°C for 30 minutes. The digestion was terminated with 2 ml of phosphate-buffered saline containing 10% fetal bovine serum, and the digested adipose tissue was centrifuged at 430 *g* for 5 minutes. After centrifugation, the top, middle, and bottom layers were assessed by a cell-staining morphometric assay, the XTT assay, and the glycerol-3-phosphate dehydrogenase assay, respectively (Fig. 1). To avoid waiting time, we performed these assays simultaneously. We also performed each assay as immediately as possible.

Cell-Staining Morphometric Assay

Respective layers after digestion and centrifugation were resuspended in phosphate-buffered saline to a total of 5 ml. First, to distinguish adipocytes from floating lipid droplets released from broken adipocytes, cells were stained with Nile Red (AdipoRed) (Cambrex, Walkersville, Md.) and Hoechst 33342 (Dojindo, Kumamoto, Japan). Cells stained with both Nile Red and Hoechst 33342 were defined as adipocytes, and cells stained only with Hoechst 33342 but negative for AdipoRed were defined as nonadipocytes. Particles stained only with AdipoRed were defined as floating lipid droplets.

Next, to distinguish viable cells from dead cells, cells were stained with Hoechst 33342 and propidium iodide (Sigma-Aldrich, St. Louis, Mo.). This staining distinguished four types of nucleated cells: viable adipocytes (positive for Hoechst 33342, negative for propidium iodide, and including lipid); dead adipocytes (positive for propidium iodide and including lipid); viable cells other than adipocytes (positive for Hoechst 33342, negative for propidium iodide, and not including lipid); and dead cells other than adipocytes (positive for propidium iodide and not including lipid). Erythrocytes, which are not stained with the nuclear staining, were not counted in this study. Samples were placed on a hematocytometer under a microscope. At least three fields (×100) were randomly photographed and the number of cells

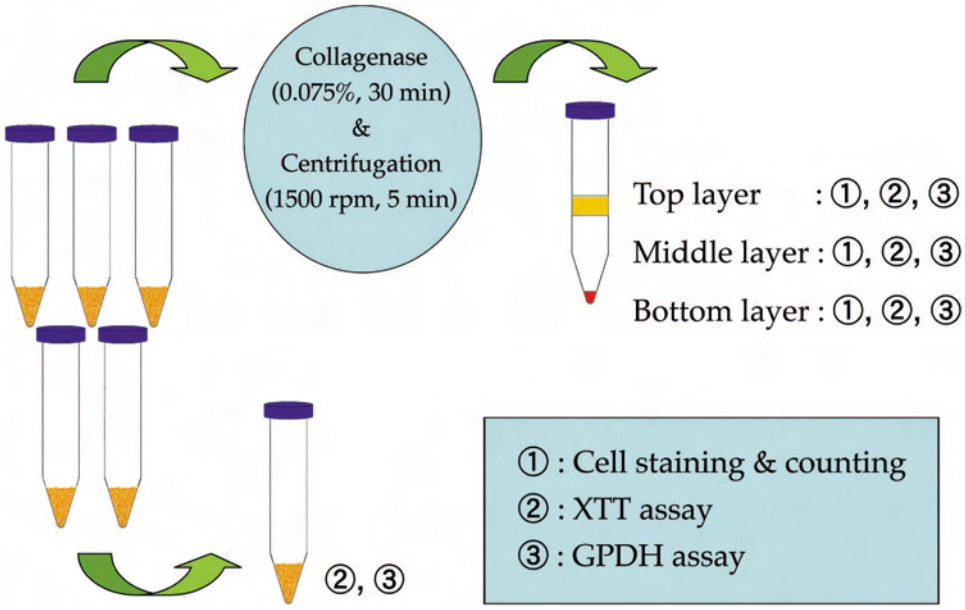

Fig. 1. A schematic diagram of tissue sampling and processing. One sample was directly subjected to the XTT assay; another sample was directly subjected to the glycerol-3-phosphate dehydrogenase assay. The other three samples were processed by digestion with collagenase and centrifugation. After centrifugation, the top, middle, and bottom layers were assessed by a cell-staining morphometric assay, the XTT assay, and the glycerol-3-phosphate dehydrogenase assay, respectively.

in each group counted. Then, the total cell number obtained from 1 g of suctioned adipose tissue was calculated.

XTT Assay

A Cell Proliferation Kit II (XTT) (Roche Diagnostics, Indianapolis, Ind.) was used according to the manufacturer's instructions. In brief, 1 g of each sample was suspended in phosphate-buffered saline containing 10% fetal bovine serum to a total of 5 ml. An XTT-labeling reagent and electron-coupling reagent were mixed at a ratio of 50:1, and 2.5 ml of this reagent mixture was added to the sample, followed by incubation in a six-well plate for 6 hours at 37°C. After the incubation, 150 μl of the solution was transferred to a 96-well plate, and the absorbance was measured at a test wavelength of 450 nm and a reference wavelength of 650 nm. The baseline absorbance of the negative control, phosphate-buffered saline containing 10% fetal bovine serum and no cells, was subtracted from each result.

To examine any correlation between the number of viable cells and the resulting values from the XTT assay for each cell type, several concentrations of adipocytes, white blood cells, and adipose-derived stem cells were prepared for assessment by the XTT assay. Adipocytes were separated from adipose tissue by digestion and centrifugation as described above, and viable adipocytes in the top layer were used. White blood cells were obtained from the peripheral blood of five healthy donors. Adipose-derived stem/stromal cells were separated from adipose tissue as described previously[6] and cultured in Dulbecco's Modified Eagle Medium containing 10% fetal bovine serum. Adipose-derived stem/stromal cells at passage 1 or 2 were used. For each cell type, samples of 1, 2, 5, and 10×10^4 viable cells/ml were prepared; the cell number was determined by cell staining with Hoechst 33342 and propidium iodide for adipocytes, and it was determined by cell counter (NucleoCounter; Chemometec, Allerod, Denmark) for white blood cells and adipose-derived stem/stromal cells.

Glycerol-3-Phosphate Dehydrogenase Assay

A GPDH Assay Kit (Cell Garage, Tokyo, Japan) was used according to the manufacturer's instructions. In brief, 1-g sample (adipose tissue or respective layers after digestion and centrifugation) was mixed with 0.25 M sucrose solution to a total of 5 ml and homogenized. The mixture was then centrifuged at 430 *g* for 5 minutes. A 1-ml aliquot

of the aqueous layer was taken and again centrifuged at 17,700 *g* for 5 minutes. The supernatant after the second centrifugation was diluted 10 times with an enzyme-extracting reagent, and the optical absorption was measured at 340 nm for 10 minutes on a 96-well plate after addition of twice the volume of a substrate reagent. Glycerol-3-phosphate dehydrogenase activity was calculated based on the following formula: Glycerol-3-phosphate dehydrogenase activity (U/ml) = $\Delta OD \times 0.482 \times 10$, where ΔOD is change in optical density per minute.

To examine correlation between the number of viable cells and the resulting values of glycerol-3-phosphate dehydrogenase activity in each cell type, we assessed several concentrations of adipocytes, white blood cells, and adipose-derived stem/stromal cells using the glycerol-3-phosphate dehydrogenase assay, following the design used for the XTT assay.

Flow Cytometry

To determine the presence of nonadipocytes, we used multicolor flow cytometric analysis to examine cells in the bottom layer, the stromal vascular fraction, for surface marker expression. The following monoclonal antibodies conjugated to fluorochromes were used: anti-CD31-PE, anti-CD34-PE-Cy7, and anti-CD45-FITC (BD Biosciences, San Jose, Calif.). Cells were analyzed with a LSR II (BD Biosciences), and cell composition percentages were determined according to surface marker expression profiles.

Statistical Analysis

Data are expressed as mean ± SEM. Comparisons of multiple groups were made using one-way analysis of variance with Bonferroni's multiple *t* test. Values of $p < 0.05$ were considered statistically significant.

RESULTS

Cell Staining

After digestion and centrifugation, many pure lipid droplets, not cells, were included together with cells in the top layer (Fig. 2). The top layer contained $4.4 \pm 0.66 \times 10^5$ viable adipocytes; $2.5 \pm 0.28 \times 10^5$ dead adipocytes; $1.5 \pm 0.23 \times 10^5$ viable cells other than adipocytes; and $7.5 \pm 0.72 \times 10^5$ dead cells other than adipocytes, which were distinguished by cell staining with Hoechst 33342 and propidium iodide (Fig. 3). The middle layer contained no cells. The bottom layer contained $10.0 \pm 2.8 \times 10^5$ viable cells other than adipocytes and $2.3 \pm 0.34 \times 10^5$ dead cells other than adipocytes (Table 1).

XTT Assay

Adipose tissue before digestion resulted in the highest value, which was similar to the sum of the three layers after digestion. The top layer produced a much higher value than that of the bottom layer, although the number of viable cells directly counted by cell staining was higher in the bottom layer (Fig. 5).

Good correlations between the number of prepared viable cells and the XTT values were respectively obtained in adipocytes, white blood cells, and adipose-derived stem/stromal cells. Adipocytes showed higher absorbance than white blood cells and adipose-derived stem/stromal cells, especially at a high concentration, differences that were statistically significant (Fig. 6). Thus, the results suggest that the value derived from the XTT assay would not be proportional to the total viable cell number when different cell types are mixed.

Glycerol-3-Phosphate Dehydrogenase Assay

Adipose tissue before digestion and centrifugation showed the highest value. After digestion and centrifugation, the value of the top layer, which was regarded as intracellular glycerol-3-phosphate dehydrogenase from floating nonruptured adipocytes, was more than twofold that of the middle layer, which was regarded as extracellular glycerol-3-phosphate dehydrogenase released from ruptured adipocytes. The bottom layer, which contained no adipocytes, showed a low value (Fig. 7).

The glycerol-3-phosphate dehydrogenase assay provided a good correlation between the number of prepared viable adipocytes and the resulting values, whereas white blood cells and adipose-derived stem/stromal cells showed no glycerol-3-phosphate dehydrogenase activity even at a high concentration (Fig. 8).

Flow Cytometry

Flow cytometric analysis revealed that the stromal vascular fraction contained CD45-positive cells, corresponding to blood-derived cells at 37 ± 5.2 percent; adipose-derived stem/stromal cells ($CD45^-CD31^-CD34^+$) at 37 ± 4.0 percent; and endothelial cells ($CD45^-CD31^+CD34^+$) at 15 ± 4.9 percent (Fig. 9 and Table 2).

DISCUSSION

In the present study, suctioned fat samples obtained from the abdomen or thighs were used. Although regional (donor-site) differences in adipose cell components may exist, a previous study reported that no statistical differences in adipose

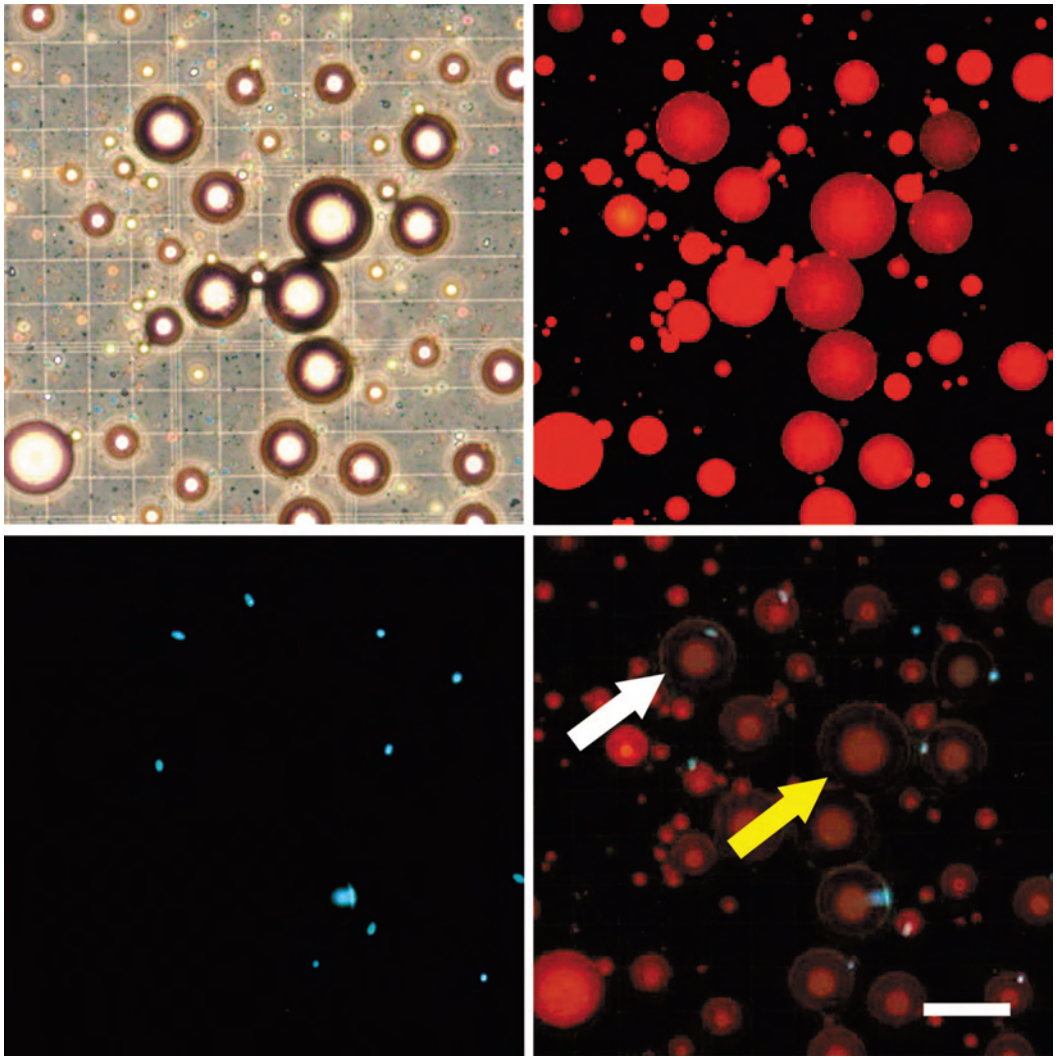

Fig. 2. Cell staining with AdipoRed and Hoechst 33342. Bright field (*above*, *left*), AdipoRed (*above*, *right*), Hoechst 33342 (*below*, *left*), and merge (*below*, *right*). A *white arrow* indicates an adipocyte. A *yellow arrow* indicates a pure lipid droplet. Scale bar = 100 μm.

tissue viability were demonstrated among abdominal fat, thigh fat, flank fat, or knee fat, based on the XTT assay.[14] In our study, no differences in adipose stromal cell yield were observed between the samples from the abdomen and the thigh (data not shown).

In staining and counting of adipocytes, distinguishing them from pure lipid droplets is necessary. In our study, many lipid droplets derived from ruptured adipocytes were observed together with adipocytes, as shown in Figures 2 through 4. Lipid droplets the same size as adipocytes (50 to 120 μm) cannot be distinguished from adipocytes without nuclear staining. In previous studies,[8,10,13] trypan blue staining was performed, but it cannot distinguish pure lipid droplets from viable adipocytes because it does not stain either of them well. The previous studies[8,10,13] reported adipocyte numbers (2.5 to 90 million/ml) which were much larger than those in our result (0.7 million/ml), and the difference may arise from the confusion of lipid droplets with adipocytes. Nuclear staining with Hoechst 33342 only is not appropriate for a viability assay, because it also stains the nuclei of dead cells. We combined propidium iodide, which stained only the nuclei of nonviable cells, with Hoechst 33342 staining, and with this combination, we distinguished four types of cells: viable adipocytes, dead adipocytes, viable cells other than adipocytes, and dead cells other than adipocytes. A combination of fluorescein diacetate and propidium iodide may be another option.[12]

We found that many nonadipocyte cells were present in the top layer after collagenase digestion

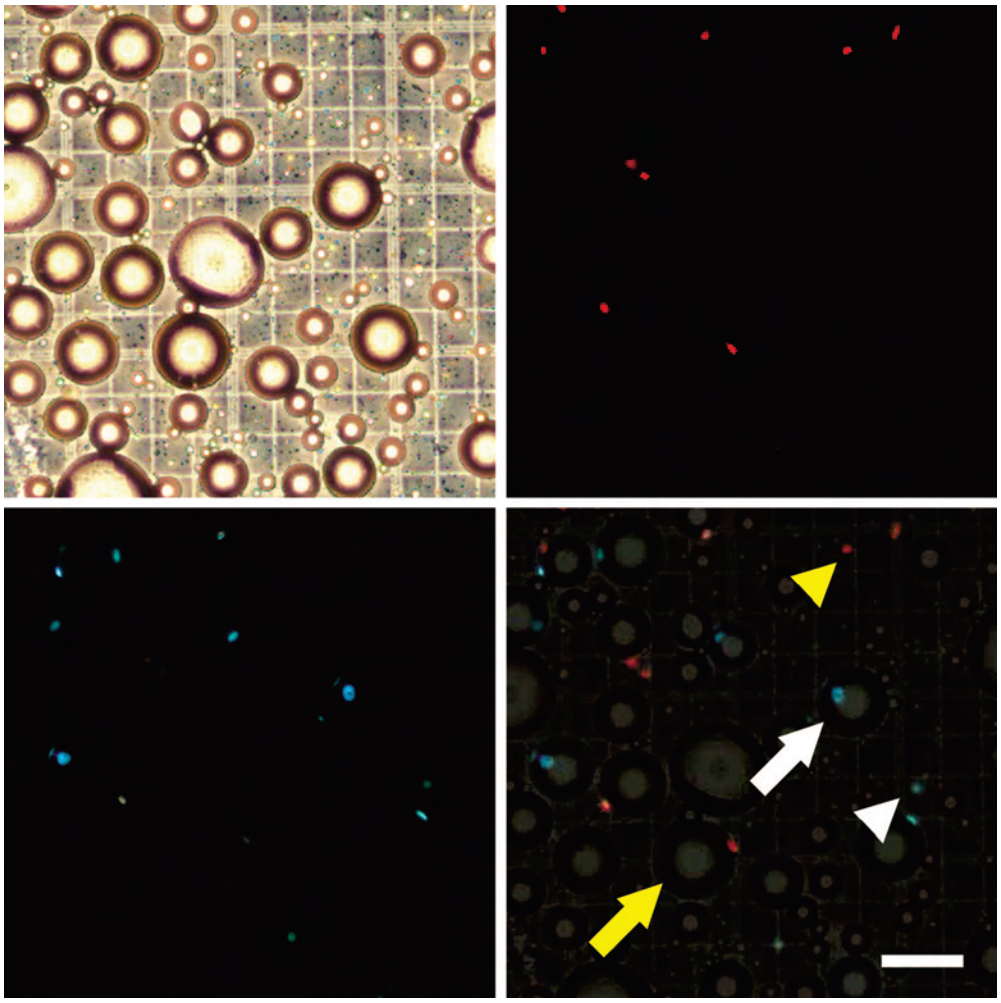

Fig. 3. Cell staining with Hoechst 33342 and propidium iodide. Bright field (*above*, *left*), propidium iodide (*above*, *right*), Hoechst 33342 (*below*, *left*), and merge (*below*, *right*). The *white arrow* indicates a viable adipocyte. The *yellow arrow* indicates a dead adipocyte. The *white arrowhead* indicates a viable cell other than adipocytes. The *yellow arrowhead* indicates a dead cell other than adipocytes. Scale bar = 100 μm.

and centrifugation, although the viable cell ratio was much lower than that of the bottom layer. Cells, especially adherent cells, may have been trapped in the floating layer and not released by centrifugation. A previous study using ceiling culture showed that cells tightly attached to mature adipocytes could generate fibroblastic cell populations with multiple differentiation potential,[20] and the cells were regarded as adipose-derived stem/stromal cells. Thus, the nonadipocyte cells detected in the top layer may contain adipose-derived stem/stromal cells and white blood cells, although more than 80 percent of the nonadipocyte cells were dead in the current study.

Table 1. Number of Cells Obtained from 1 g of Adipose Tissue (×10^5)*

	Adipocytes		Other Cells	
Layer	Viable	Dead	Viable	Dead
Top	4.4 ± 0.66	2.5 ± 0.28	1.5 ± 0.23	7.5 ± 0.72
Middle	0	0	0	0
Bottom	0	0	10.0 ± 2.8	2.3 ± 0.34

*Data are expressed as mean ± SEM (n = 10).

Among colorimetric assays of cell viability, the XTT and MTT assays have been widely used. We first assessed the viability of adipose tissue using both the XTT and MTT assays, but the MTT assay did not give reproducible results (data not shown), unlike the XTT assay. In the MTT assay, the resulting colored formazan product is insoluble and requires a dissolving procedure,[19] which may impair reproducibility, especially when using adipose tissue.

We also found that the resulting values of the XTT assay differed among cell types. This difference is likely the reason for the higher value from the XTT assay in the top layer compared with the bottom layer; the results from the cell-staining assays made it clear that the bottom layer contained a much larger number of viable cells. Adipocytes exhibited much higher absorbance in the XTT assay compared with white blood cells and adipose-derived stem/stromal cells. This difference may be attributable to the increase in mitochondrial capacity (number of mitochondrion profiles, concentration of numerous mitochondrial proteins, and oxygen consumption rate) during adipogenesis.[21] Some authors have reported in previous studies[14,16] that the XTT assay is well suited to assessing adipose tissue viability; however, it is not specific for adipocytes, and careful interpretation of results is needed when contamination by other cell types is present. Our results demonstrate that the bottom layer, which contains no adipocytes, had a substantial XTT value. Given this finding, it is possible that in previous studies, vascular stromal fraction cells such as white blood cells, adipose stromal cells, and vascular endothelial cells were mistakenly counted as adipocytes.

Glycerol-3-phosphate dehydrogenase is normally intracellular, and extracellular glycerol-3-phosphate dehydrogenase is considered proportional to the amount of adipocyte destruction.[9,18]

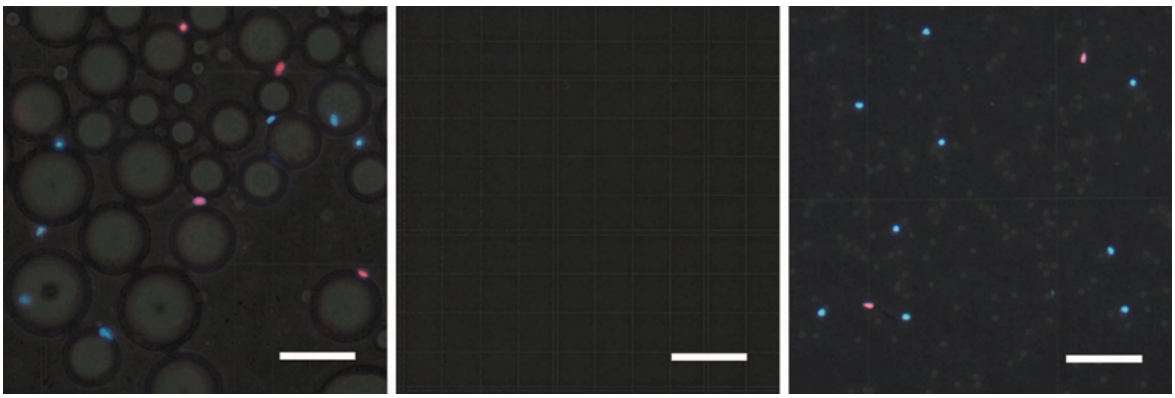

Fig. 4. Representative views of each layer stained with Hoechst 33342 and propidium iodide. In the top layer, four types of cells (viable adipocytes, dead adipocytes, viable cells other than adipocytes, and dead cells other than adipocytes) and pure lipid droplets were observed. No cells were detected in the middle layer, whereas nonadipocytes, viable or dead, were seen in the bottom layer. Scale bar = 100 μm.

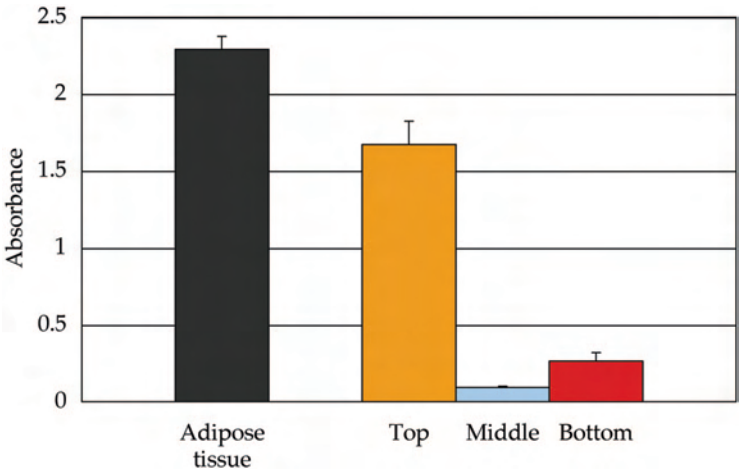

Fig. 5. Results of the XTT assay for 1 g of suctioned adipose tissue and respective layers after digestion and centrifugation (n = 10). Adipose tissue before digestion resulted in the highest value, which was similar to the sum of the three layers after digestion. Statistical significance was detected between any combinations of groups. Values are mean ± SEM.

Therefore, examining total glycerol-3-phosphate dehydrogenase in adipose tissue is not appropriate for viability assessment. To gain a full understanding of the results of previous studies, we would have to confirm whether the reported glycerol-3-phosphate dehydrogenase value was intracellular or extracellular or a mixture of both. In our study, the glycerol-3-phosphate dehydrogenase activity of the top layer was regarded as intracellular glycerol-3-phosphate dehydrogenase from nonruptured adipocytes, although this layer appeared to contain both viable and dead (but not ruptured) adipocytes. These latter adipocytes may die because of the effects of collagenase, although they did not rupture and the intracellular contents were not released into the middle layer. The glycerol-3-phosphate dehydrogenase activity of the middle layer indicated that approximately 30 percent of total adipocytes were ruptured. The rate of this extracellular glycerol-3-phosphate dehydrogenase can change, depending on adipose tissue sampling methods[22] and the digestion procedure.[18] The glycerol-3-phosphate dehydrogenase assay is superior to the XTT assay because of its strict

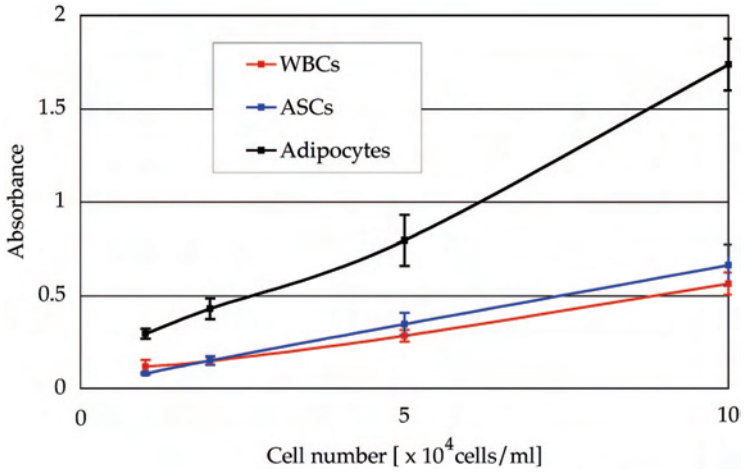

Fig. 6. Correlation between the number of prepared viable cells and the resulting values of the XTT assay ($n = 5$). Good correlations were obtained in adipocytes, white blood cells (*WBCs*), and adipose-derived stem/stromal cells (*ASCs*). Values are mean ± SEM.

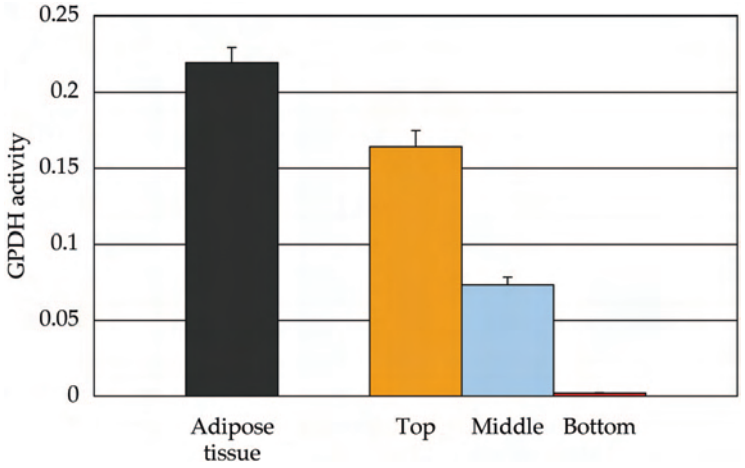

Fig. 7. Results of the glycerol-3-phosphate dehydrogenase assay for 1 g of adipose tissue and respective layers after digestion and centrifugation ($n = 10$). Adipose tissue before digestion and centrifugation showed the highest value. The bottom layer, which contained no adipocytes, showed a low value. Statistical significance was detected between any combinations of groups. Values are mean ± SEM.

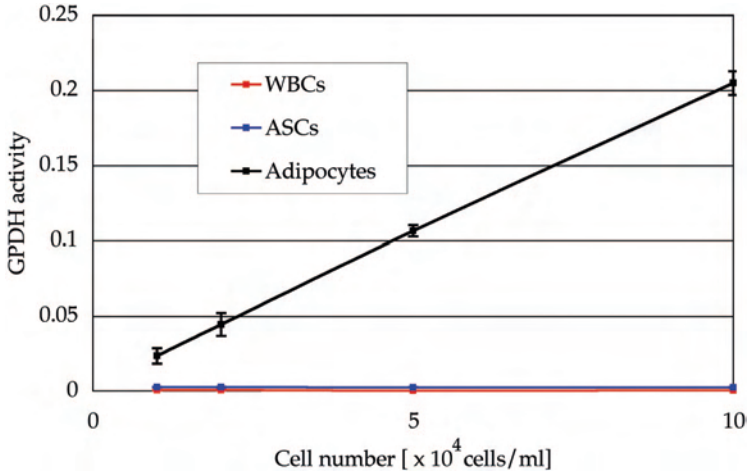

Fig. 8. Correlation between the number of prepared viable cells and the resulting values of the glycerol-3-phosphate dehydrogenase assay ($n = 5$). The glycerol-3-phosphate dehydrogenase assay provided a good correlation in adipocytes. White blood cells (*WBCs*) and adipose-derived stem/stromal cells (*ASCs*) showed no glycerol-3-phosphate dehydrogenase activity even at a high concentration. Values are mean ± SEM.

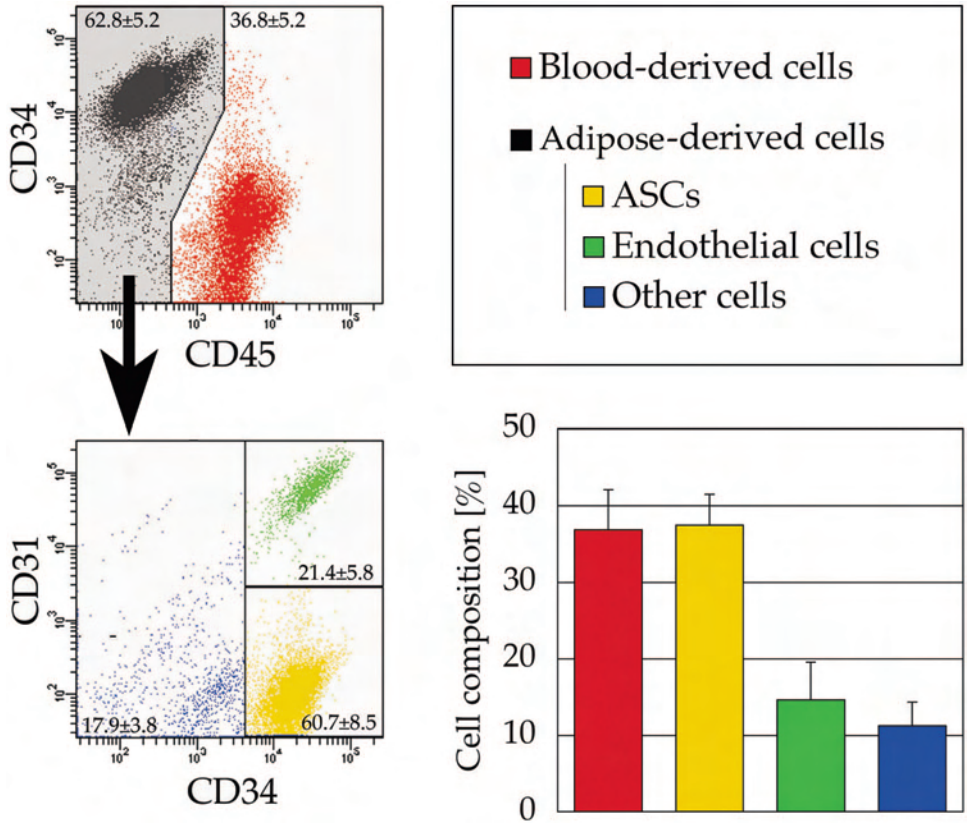

Fig. 9. Multicolor flow cytometric analysis of cells in the bottom layer. $CD45^+$ cells were regarded as blood-derived cells. $CD45^-$ cells were regarded as adipose-derived cells and were processed to the next analysis. $CD45^-CD31^-CD34^+$ cells were regarded as adipose-derived stem/stromal cells (*ASCs*), whereas $CD45^-CD31^+CD34^+$ cells were regarded as endothelial cells.

Table 2. Cell Composition in the Bottom Layer*

		Adipose-Derived Cells		
	Blood-Derived Cells	ASCs	Endothelial Cells	Other Cells
CD45	+	−	−	−
CD31		−	+	−
CD34		+	+	−
Percentage in all cells	36.8 ± 5.2		62.8 ± 5.2	
Percentage in adipose-derived cells		60.7 ± 8.5	21.4 ± 5.8	17.9 ± 3.8
Calculated percentage in all cells	36.8 ± 5.2	37.4 ± 4.0	14.6 ± 4.9	11.2 ± 3.1

ASCs, adipose-derived stem/stromal cells; +, positive for the cell surface marker; −, negative for the cell surface marker.
*Data are expressed as mean ± SEM ($n = 5$).

specificity for adipocytes, which the current findings support. Although the previous studies[9,10,18] appropriately used the glycerol-3-phosphate dehydrogenase assay, a control curve using premeasured adipocytes or a combination of direct cell counting is needed to evaluate the absolute number of adipocytes.

From 1 g of suctioned adipose tissue, we obtained 6.9×10^5 adipocytes (viable-to-dead ratio, 4.4:2.5) and 2.1×10^6 nonadipocyte cells (viable-to-dead ratio, 11.5:9.8). Based on the glycerol-3-phosphate dehydrogenase data for the top and middle layers, we speculate that 1 g of suctioned adipose tissue originally contains approximately 1 million adipocytes (6.9×10^5 adipocytes in the top layer and approximately 3×10^5 adipocytes in the middle layer). Although the size of adipocytes may differ according to nutritional status,[23] previous studies[24,25] reported relatively similar adipocyte sizes, ranging from 50 to 150 μm, with a mean size between 90 and 100 μm regardless of the severity of obesity. Assuming that adipocytes are cubes (10^6 μm^3) or spheres (5.23×10^5 μm^3) with a side or diameter of 100 μm, the number of adipocytes in 1 ml adipose tissue would correspond to 0.95 million or 1.81 million, respectively, under the condition that adipocytes occupy 95 percent of adipose tissue volume; thus, the theoretical number of adipocytes in the 1 ml of adipose tissue is likely to be between the two numbers. This suggestion is supported by the results of a previous study[23] indicating that adipocyte number per 1 g of adipose tissue is 1.51 ± 0.47 million (mean ± SD). Because the adipocyte number of noncentrifuged suctioned adipose tissue is likely to be smaller than that of intact adipose tissue,[26] the estimated number in our study (1 million per 1 ml of suctioned adipose tissue) seems to be reasonable.

Multicolor flow cytometry revealed that 37 percent of nonadipocyte cells in stromal vascular fraction (the bottom layer) are adipose-derived stem/stromal cells, a ratio consistent with our previous findings.[5] Assuming that the nonadipocyte cell composition in the top layer is similar to that of the bottom layer, we can speculate that the number of adipose-derived stem/stromal cells in 1 g of suctioned adipose tissue is approximately half that of adipocytes. Considering also that the number of adipose-derived stem/stromal cells in suctioned adipose tissue is significantly smaller than in intact adipose tissue,[6] the adipose-derived stem/stromal cell number in intact adipose tissue may be similar to that of adipocytes. In addition, it may be much larger than that described in a previous histologic study,[27] which reported percentages of adipocytes and stromal cells in the adipose tissue as 89 to 98 percent and 2 to 10 percent, respectively. In a histologic assessment of the adipose tissue, a single 100-μm adipocyte, for example, could appear in 12 serial sections (8 μm thick; 96 μm in all), but each section contains different adipose-derived stem/stromal cells; thus, a single adipocyte could be repeatedly counted by mistake, and a careful evaluation of the specimens is necessary for counting cell numbers. Therefore, thicker sections, as thick as 200 μm, combined with an appropriate calculation formula such as that used in a previous study[25] may contribute to avoiding mistakes in cell counting.

CONCLUSIONS

Single use or a combination of the viability assays used in this study can accurately determine the number of viable adipocytes and other cells, although other assays such as the adenosine triphosphate assay[28] and glucose transport experiment[29] should be further investigated for applications to adipose tissue. The major limitation of the current study is that the assays are performed only after enzyme digestion and centrifugation, which partly destroys adipocytes and other cells. Thus, directly assessing the original adipocytes and other cellular components without tissue dissociation is difficult. A three-di-

mensional measurement that can allow analysis without tissue dissociation should be developed for a more accurate anatomical evaluation of adipose tissue.

Kotaro Yoshimura, M.D.
Department of Plastic Surgery
University of Tokyo School of Medicine
7-3-1, Hongo, Bunkyo-Ku
Tokyo 113-8655, Japan
yoshimura-pla@h.u-tokyo.ac.jp

ACKNOWLEDGMENT

This study was financially supported by Grants-in-Aid contract no. B2-19390452 from the Japanese Ministry of Education, Culture, Sports, Science, and Technology.

REFERENCES

1. Rosen, E. D., and Spiegelman, B. M. Adipocytes as regulators of energy balance and glucose homeostasis. *Nature* 444: 847, 2006.
2. Katz, A. J., Llull, R., Hedrick, M. H., and Futrell, J. W. Emerging approaches to the tissue engineering of fat. *Clin. Plast. Surg.* 26: 587, 1999.
3. Zuk, P. A., Zhu, M., Mizuno, H., et al. Multilineage cells from human adipose tissue: Implications for cell-based therapies. *Tissue Eng.* 7: 211, 2001.
4. Casteilla, L., and Dani, C. Adipose tissue-derived cells: From physiology to regenerative medicine. *Diabetes Metab.* 32: 393, 2006.
5. Yoshimura, K., Shigeura, T., Matsumoto, D., et al. Characterization of freshly isolated and cultured cells derived from the fatty and fluid portions of liposuction aspirates. *J. Cell. Physiol.* 208: 64, 2006.
6. Matsumoto, D., Sato, K., Gonda, K., et al. Cell-assisted lipotransfer: Supportive use of human adipose-derived cells for soft tissue augmentation with lipoinjection. *Tissue Eng.* 12: 3375, 2006.
7. Gimble, J. M., Katz, A. J., and Bunnell, B. A. Adipose-derived stem cells for regenerative medicine. *Circ. Res.* 100: 1249, 2007.
8. Piasecki, J. H., Gutowski, K. A., Lahvis, G. P., and Moreno, K. I. An experimental model for improving fat graft viability and purity. *Plast. Reconstr. Surg.* 119: 1571, 2007.
9. Wolter, T. P., von Heimburg, D., Stoffels, I., et al. Cryopreservation of mature human adipocytes: In vitro measurement of viability. *Ann. Plast. Surg.* 55: 408, 2005.
10. Pu, L. L. Q., Cui, X., Fink, B. F., et al. The viability of fatty tissues within adipose aspirates after conventional liposuction: A comprehensive study. *Ann. Plast. Surg.* 54: 288, 2005.
11. Tholpady, S. S., Aojanepong, C., Llull, R., et al. The cellular plasticity of human adipocytes. *Ann. Plast. Surg.* 54: 651, 2005.
12. Moscatello, D. K., Dougherty, M., Narins, R. S., and Lawrence, N. Cryopreservation of human fat for soft tissue augmentation: Viability requires use of cryopreservation and controlled freezing and storage. *Dermatol. Surg.* 31: 1506, 2005.
13. Boschert, M. T., Beckert, B. W., Puckett, C. L., and Concannon, M. J. Analysis of lipocyte viability after liposuction. *Plast. Reconstr. Surg.* 109: 761, 2002.
14. Rohrich, R. J., Sorokin, E. S., and Brown, S. A. In search of improved fat transfer viability: A quantitative analysis of the role of centrifugation and harvest site. *Plast. Reconstr. Surg.* 113: 391, 2004.
15. Atik, B., Ozturk, G., Erdogan, E., and Tan, O. Comparison of techniques for long-term storage of fat grafts: An experimental study. *Plast. Reconstr. Surg.* 118: 1533, 2006.
16. Smith, P., Adams, W. P., Lipschitz, A. H., et al. Autologous human fat grafting: Effect of harvesting and preparation techniques on adipocyte graft survival. *Plast. Reconstr. Surg.* 117: 1836, 2006.
17. MacRae, J. W., Tholpady, S. S., Ogle, R. C., and Morgan, R. F. Ex vivo fat graft preservation: Effects and implications of cryopreservation. *Ann. Plast. Surg.* 52: 281, 2004.
18. Lalikos, J. F., Li, Y., Roth, T. P., et al. Biochemical assessment of cellular damage after adipocyte harvest. *J. Surg. Res.* 70: 95, 1997.
19. Roehm, N. W., Rodgers, G. H., Hatfield, S. M., and Glasebrook, A. L. An improved colorimetric assay for cell proliferation and viability utilizing the tetrazolium salt XTT. *J. Immunol. Methods* 142: 257, 1991.
20. Miyazaki, T., Kitagawa, Y., Toriyama, K., et al. Isolation of two human fibroblastic cell populations with multiple but distinct potential of mesenchymal differentiation by ceiling culture of mature fat cells from subcutaneous adipose tissue. *Differentiation* 73: 69, 2005.
21. Wilson-Fritch, L., Burkart, A., Bell, G., et al. Mitochondrial biogenesis and remodeling during adipogenesis and in response to the insulin sensitizer Rosiglitazone. *Mol. Cell. Biol.* 23: 1085, 2003.
22. Shiffman, M. A., and Mirrafati, S. Fat transfer techniques: The effect of harvest and transfer methods on adipocyte viability and review of the literature. *Dermatol. Surg.* 27: 819, 2001.
23. van Harmelen, V., Skurk, T., Röhrig, K., et al. Effect of BMI and age on adipose tissue cellularity and differentiation capacity in women. *Int. J. Obes.* 27: 889, 2003.
24. Garaulet, M., Hernandez-Morante, J. J., Lujan, J., et al. Relationship between fat cell size and number and fatty acid composition in adipose tissue from different fat depots in overweight/obese humans. *Int. J. Obes. (Lond.)* 30: 899, 2006.
25. Sjöström, L., Björntorp, P., and Vrána, J. Microscopic fat cell size measurements on frozen-cut adipose tissue in comparison with automatic determination of osmium-fixed fat cells. *J. Lipid Res.* 12: 521, 1971.
26. Kurita, M., Matsumoto, D., Shigeura, T., et al. Influences of centrifugation on cells and tissues in liposuction aspirates: Optimized centrifugation for lipotransfer and cell isolation. *Plast. Reconstr. Surg.* 121: 1033, 2008.
27. Rink, J. D., Simpson, E. R., Barnard, J. J., and Bulun, S. E. Cellular characterization of adipose tissue from various body sites of women. *J. Clin. Endocrinol. Metab.* 81: 2443, 1996.
28. Pohjala, L., Tammela, P., Samanta, S. K., et al. Assessing the data quality in predictive toxicology using a panel of cell lines and cytotoxicity assays. *Anal. Biochem.* 362: 221, 2007.
29. Moore, J. H., Kolaczynski, J. W., Morales, L. M., et al. Viability of fat obtained by syringe suction lipectomy: Effects of local anesthesia with lidocaine. *Aesthetic Plast. Surg.* 19: 335, 1995.