A NEW SCANNING ELECTRON MICROSCOPE METHOD FOR DETERMINATION OF ADIPOCYTE SIZE IN HUMAN FAT TISSUE: CORRELATION WITH WEIGHT LOSS AND FAT DEPOT BURDEN.

W Guo, S Bigornia, W Xie, Lisa Sullivan, M McDonnell, K Clemente, I Leizerman, T Pirtskhalava, JL Kirkland, N Gokce, BE Corkey, C Apovian

Boston University School of Medicine

Abstract

Objective: Compare the use of an electron microscopy method (EM), WETSEM™, in the sizing of fat cells to that of the collagenase digestion (CO) method. Wet SEM utilizes capsules to protect wet samples from the high vacuum needed for EM. Methods: Subcutaneous fat was harvested from subjects at baseline and at 6 weeks of participating on a similar dietary intervention. Fat cell size determined by wet SEM was compared to those determined by CO. Results: There is a close correlation in cell size distribution measured by the two methods. Using the wet SEM method, we also detected a decrease in fat cell size in association with weight loss. Conclusions: Wet SEM can be a useful tool for in situ fat tissue analysis, and may be more accurate than current standards.

Introduction

•Obesity is associated with increased fat cell number or increased fat cell size.

•Larger abdominal fat cells are associated with insulin resistance and increased risk of developing diabetes.

•Impaired preadipocyte replication and differentiation may contribute to cellular hypertrophy of existing fat cells, which is also linked to increased risk for diabetes.

•Reliable and convenient methods for determining fat cell size are important in defining the basis of metabolic diseases.

•Collogenase digestion is the most widely used protocol for fat cell size determination, however it has several short comings: 1) inconvenience for large scale tissue handling due to the need for fresh tissue 2) large adipocytes tend to rupture and coalesce into fat droplets making the process of distinguishing between intact and broken cells difficult.

•To address limitations of existing methods, a novel scanning electron microscopy (SEM) method called WETSEM™ [1] was investigated to rapidly measure adipocyte size using intact fat tissue. This method has several advantages: 1) Sample capsules are used to protect wet samples from the high vacuum needed for EM imaging 2) Sample imaging with these capsules facilitates viewing cellular distribution with clarity in the context of tissue architecture 3) Permits preservation of tissue samples for subsequent analyses, unlike collogenase digestion.

•In parallel with the wet SEM analysis, we prepared fat cell suspensions of adipose tissue samples from identical subjects using the collagenase digestion method and determined fat cell size by photomicroscopy followed by direct diameter measurement (the CO method).

•Objective: To compare the wet SEM and CO methods for assessment of adipocyte size.

Methods

Patient characteristics:

•A total of ten obese female subjects were recruited through the Nutrition and Weight Management Clinic at Boston Medical Center.

Table 1. Subject characteristics.

Subj.	age (yr)/race	basal BMI	BM (lb) before diet	BM (lb) after diet	FM (lb) before diet	FM (lb) after diet
P11	42/wht/cau	54.4	292	287	136	135
P13	39/blk/aa	46.5	237	226	109	100
P15	32/blk/aa	45.4	282	264	137	130
P16	33/Wht/his	38.7	251	234	107	101
P17	57/wht/cau	48.9	258	244	125	115
P2	21/blk/aa	106.3	520	562	--	--

Hypocaloric diet consisting of 38% CHO, 28% pro, 34% fat. Subjects were on this diet with optional 400 kcal nonfat food supplements for 6 weeks. Abdominal subcutaneous fat tissue biopsies were taken before and after weight loss.
**blk: 'black, car: Caribbean, cau: Caucasian, aa: Africa American, his: Hispanic Latino; all subjects were female.

Subcutaneous biopsy:

•Abdominal subcutaneous fat biopsies were obtained in skinfold regions lateral to the umbilicus, using sterile technique and 2% lidocaine.

•Punch biopsy and cannula needle aspiration employed to provide intact and broken adipose cells respectively.

•Samples stored in formalin for SEM or directly used for collagenase digestion.

Scanning Electron Microscopy of intact, hydrated samples (wet SEM):

•Allows direct observation of fully hydrated biological samples (cells, tissue biopsies) using standard scanning electron microscopy.

•A capsule that holds the hydrated sample isolates it from the vacuum in the microscope chamber, as described previously [1].

•The capsule contains a thin membrane, which is transparent to the electron beam, thereby allowing it to interact with the sample.

•The technology is suitable for lipid imaging due to the contrast mechanism, which is sensitive to small variations in atomic number. Thus, the contrast between carbon, which is abundant in lipids and the rest of the cell (mostly oxygen) is sufficient to be readily detected without staining.

•Formalin-fixed tissue biopsies of 3 mm diameter and up to 1 mm in thickness were trimmed with a razor blade to fit the internal dimension of the sample capsules

•Each sample was placed in a capsule and then the capsule was sealed and placed in the scanning electron microscope chamber.

•Vacuum was applied and images were taken using back scattered electron detection.

•Cell size distribution measured using AnalySIS software were analyzed based on at least 200 cells per sample.

Direct size determination in a fat cell suspension (Collagenase digestion):

•Approximately 0.5 gm fat tissue was finely minced and digested with collagenase (1 mg/ ml) dissolved in Krebs-Ringer's phosphate buffer solution with 2% BSA in a shaking water bath (37°C, 125 Hz) for 30 min.

•After being washed with KRP solution 3 times, cells were re-suspended in KRP for photomicrography. Photographs were taken using an inverted microscope equipped with a Nikon CCD with original magnification of 10 x.

•The cell diameter (d) was measured using Spot Advanced program in reference to the fixed ratio ([10α]: 1394 pixels = 1mm) provided by the manufacturer and cell area was calculated as $\pi(d/2)^2$. At least 140 fat cells were analyzed in this way for each fat cell suspension.

DEXA analysis:

•Whole-body fat mass and fat-free mass were assessed by Dual-energy X-ray absorptiometry using QDR for Windows™ software.

•Transverse scans were used for the measurement of fat and lean tissue mass, and pixels of soft tissue were used to calculate the ratio (R value) of mass attenuation coefficients at 38 and 70 ke V, using software version 3.9.4.

Statistics:

•Tests for differences in cell size between methods. Tests were performed by patient, using the Wilcoxon signed rank test which tests for differences in medians between methods. This nonparametric test was used as it does not assume that data is derived from a normal distribution.

Results

1. Comparison of wet SEM images with those of isolated fat cell suspensions.

•As illustrated in Fig. 1A, a wet SEM image contains about 50 individual grids. Within each grid, one can clearly visualize the cellular ultrastructure (Fig. 1B), and individual fat cells can be identified by their intact cytosolic rims. Since many of these cells do not show the perfect round shapes found in isolated fat cell suspensions (Fig. 1C), their sizes were determined by manually marking the cell borders, using AnalySIS software, which then calculates the area per cell. Figure 1D shows a photo of intact fat tissue under light microscope.

2. Comparison of cell size distribution determined by SEM and by CO methods.

•As shown in Fig. 2, the majority of the fat cell sizes fall in the range of 5,000-20,000 μm^2, with peak area near 10,000 μm^2, as in previous reports [2].

•The precise pattern was dependent on the individual subject and appears to be independent of BMI (see Table 1).

•Among the four subjects we studied, mean results obtained from SEM were similar to those obtained from the CO method (Fig. 2A-D).

Table 1. Tests for difference between methods. There appears to be a difference between patients 15 and 17. Those differences could be inherent to the methods used, or could be introduced by method-independent factors, since the two measurements were performed on different tissue samples (from the same subject) and by different investigators in different laboratories. Nonetheless, results indicate that the differences between cell sizes obtained with the two methods are relatively small, and the wet SEM method might be a useful substitute for the CO method as a tool for studying fat cell size distribution.

Patient	P for equality of Medians
2	0.3812
15	0.0001
16	0.8349
17	0.0040

Results Continued

3. SEM analysis of the effects of body fat mass change on fat cell size distribution in subcutaneous fat.

•We examined fat cell size distribution in subjects before and after a change in body fat mass or weight (subjects ≥ 300 lbs could not complete DEXA scan).

•To do this, 6 subjects were put on a hypocaloric diet for 6 weeks. As a result, body fat mass decreased in 5 subjects as verified by DEXA analysis (Table 1). Subcutaneous adipose tissue biopsies were obtained before and after the dietary intervention and analyzed using the wet SEM method.

•Cell size distributions shown in Fig. 3, indicate that for the subjects who gain weight, there was a substantial increase in larger fat cells (patient 2). For those who lost 1-10 lbs of fat mass, there was a different extent of shift from large fat cell size to smaller ones, in accord with previous studies [3].

•The differential response of fat cell sizes to fat mass (weight) became clearer when we plotted the number of fat cells in low (1-10,000 μm^2), medium (10,001-20,000 μm^2) and large (20,001-30,000 μm^2) size ranges.

•As shown in Fig. 4, for subjects with large fat cells at baseline (Fig. 4, A-C), weight loss caused a shift of fat cells to the lower size range (1-10000 μm^2). On the other hand, for those who originally had smaller fat cells (mostly in the range of 1-10000 μm^2, Fig. 4, D-E), there was little change in the distribution pattern, despite a >5 % loss of fat mass. For the subject who gained weight after the dietary intervention, possibly due to failure to comply with the diet, an increase in large fat cells was detected (Fig. 4F).

Figure 1. Ultarastructure of formalin-fixed fat tissue obtained by SEM image at low (A) and high (B) magnification, and light photomicrograph of a fat cell suspension after collagenase digestion (C).

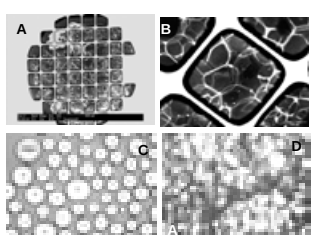


Figure 2. Comparison of subcutaneous fat cell size distributions obtained by SEM method (squares) with those obtained by the collagenase digestion method (circles) for four individual subjects (A-D). Results are shown as percentiles of cells distributed within a 2000 μm^2 range with increments from 0 to 35,000 μm^2.

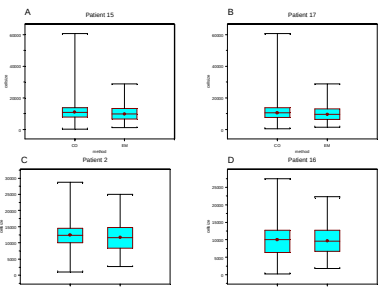


Figure 3. Comparison of depot dependent fat cell size distributions obtained by SEM method (squares) with that by CO method (circles). Results are shown as percentiles of cells distributed within a 2000 μm^2 range with increments from 0 to 35,000 μm^2.

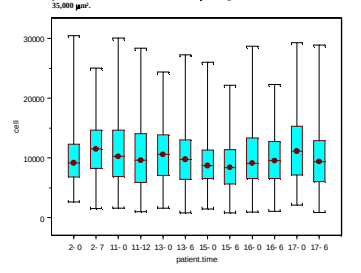
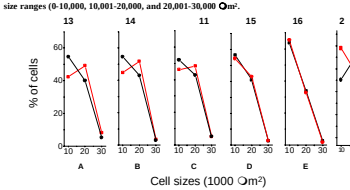


Figure 4. SEM analysis of fat cell size distributions before and after diet-induced body fat mass changes in six subjects plotted as the percentages of cells that fall into one of the three size ranges (0-10,000, 10,001-20,000, and 20,001-30,000 Qm²).



Discussion

•Over 4000 fat cells from a combination of subjects using both wet SEM and CO methods independently were examined.

•We demonstrated a close correlation in fat cell size measurements in human fat tissue biopsies analyzed by these two methods, indicating that the wet SEM method can be used as a valid tool to replace conventional time- and labor-consuming methods for fat cell size analysis from adipose biopsy tissue samples.

•Since the wet SEM method requires very small sample sizes (3-5 mm^3), it is especially suitable for situations when the fat tissue sample is not of sufficient size for other methods.

•Since samples are not destroyed using wet SEM , subsequent biochemical and immunohistological analysis can be performed.

•Because of their large size, fat cells are visible by light microscopy. A comparison of the images obtained by light microscopy (Fig. 1D) and by wet SEM (Fig. 1A&B) clearly demonstrates that SEM is advantageous because of the well defined cytosolic rims and cellular ultrastructural characteristics allowing for more accurate identification of intact adipose cells from ruptured ones.

•SEM results are similar to those obtained by standard histology methods, but the procedure is simpler and the analysis time is shortened from days to minutes. The method also avoids accidental tissue distortion that commonly occurs during sample preparation for light microscopic histological analysis.

References

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