PROCEEDINGS OF THE SOCIETY FOR EXPERIMENTAL BIOLOGY AND MEDICINE 156, 251-256 (1977)

A Reliable Photomicrographic Method for Determining Fat Cell Size and Number: Application to dietary obesity[1] (39916)

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In studies of adipose tissue, in both normal and obese subjects, morphology is becoming increasingly important to quantify. While several methods have been described to determine fat cell diameter, two are most widely used: that of Hirsch and Gallian (1), based on the electronic counting of osmium-fixed fat cells in a saline suspension, and that based on the direct microscopic measurement of the diameter of collagenase-isolated fat cells (2).

The expense of the equipment and reagents for the osmium technique often renders it impractical for use. The microscopic method is economical, but the time required per sample to size fat cells under the microscope (about 30 min) makes it inappropriate to perform on more than a very few samples and is further complicated by fat cell lysis upon standing.

We report here a photomicrographic method derived from the latter, except that the time of handling isolated fat cells is minimized by measuring adipocyte diameters not under the microscope, but on printed and enlarged photomicrographs. The results we obtained using this method, with both human and rat tissue, are compared with those we obtained using the currently accepted osmium fixation method of Hirsch and Gallian (1). In addition, we report the effect on morphology of epididymal adipose cells, as measured utilizing the photomicrograph and method, of short- and long-term feeding of a high-fat diet to rats.

Materials and methods. (I) *Cell morphology techniques.* Adipose tissue was obtained at sacrifice from the epididymal fat pads of Wistar rats or from normal-weight and obese human subjects who were undergoing elective surgery. Three of the human subjects were grossly obese patients undergoing intestinal bypass surgery. Small fragments of tissue were taken from the entire rat fat pad or human biopsy sample so as to obtain several representative samples. Tissue samples were washed with isotonic saline at 37° in order to free them of oil droplets, as described by Hirsch and Gallian (1). The samples were blotted on absorbent paper, weighed, and processed according to the following procedures.

(*a*) *Osmium fixation technique.* Both human and rat tissues were incubated for 48 hr in plastic scintillation vials (70–100 mg of tissue per flask) containing 15 ml of 2% OsO_4 in 0.05 *M* collidine buffer. The osmium-fixed fat cells were then washed, suspended in 500 ml of saline, and the number of cells in 2 ml was electronically counted using a Coulter counter (1). Lipids (300 mg of tissue) were extracted twice with 15 ml of $CHCl_3/CH_3OH$, 2:1 (v/v), and determined gravimetrically. The mean cell size was expressed as the micrograms of lipid per cell.

A comparison of fat cell size distribution between the photomicrographic method and electronic sorting of osmium-fixed fat cells was also made on four representative rat adipose tissue samples. Fat cell sizes were determined directly, as described below, for the photomicrographic method, and the

[1] This study was supported by grants from the National Institutes of Health (Obesity Center AM 17624 and NIH 08965) and the Nutrition Foundation (2577).

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ISSN 0037-9727

comparison between this direct measurement and the electronic method was performed to determine the usefulness of the electronic method for assessing cell size distributions. The electronic sorting of osmium-fixed fat cells was performed by using a Coulter counter, adapted with an EE_1 timer, using a 400-μm aperture, and calibrated with corn pollen. Cell diameters were electronically selected by varying in a predetermined manner the amplification and aperture current settings so that only cells of a certain diameter range were counted.

(*b*) *Photomicrographic technique.* Isolated fat cells were prepared as described by Rodbell (3), with the modifications of Martinsson (4). Approximately 300 mg of adipose tissue were incubated in plastic vials containing 3 ml of Krebs–Ringer bicarbonate buffer, pH 7.4, at 37°, with 15 μmole of glucose, 120 mg of bovine serum albumin, fraction V (Pentex), and 5 mg of collagenase (Worthington Biochemicals Corporation, Freehold, N.J.). The flasks were gassed for 3 min with 95% O_2–5%CO_2 and then incubated for 1 hr, with agitation by hand every 10 min. Fat cells were separated from the stroma by passage through a nylon mesh filter (250 μm) and washed once with incubation medium free of collagenase at 37°. No centrifugation of cells was performed. Most of the infranatant solution was removed in order to get a concentrated cell suspension. The cell preparation was then stirred gently and 50 μl of the stirred cell suspension was placed on a glass slide. Photographs were taken immediately through a Zeiss photomicroscope using a yellow light and a total magnification in the film plane between 25 and 40, depending on the size of the cells. Under these conditions the depth of field[3] was large as compared with the mean cell radius of the fat cell populations, so that all cells, regardless of their size, appeared clearly defined, indicating that their equatorial planes were in focus (Fig. 1). Three glass slides of the fat

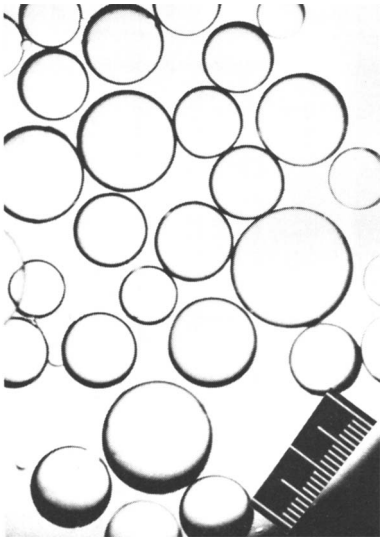

FIG. 1. Human isolated adipocytes as they were photographed under the microscope (scale, 100 μm).

cell suspension were prepared and four photographs were taken of each slide. A micrometer scale was photographed after every tenth picture. Photomicrography took 3 to 4 min per cell suspension. After developing, instant printing of the microfilm was possible using a 3M microfilm enlarger. Determination of the diameter was done using a semiautomatic counter developed by Joliff (6). Measurements were made by placing a transparent scale along the largest diameter of the cells. This scale was connected to a frequency counter and each measurement was recorded automatically in 10-μm classes between 0 and 200 μm. This device permitted measurement of 200 cells in less than 10 min. The mean fat cell diameter and standard deviation were calculated using the programs of Lowy and Manchon (7) for a Programma Olivetti 101. The mean fat cell volume was calculated with Goldrick's formula (8) and the mean fat cell weight was determined by using triolein density (0.915).

(*II*) *The effect of short- and long-term high-fat feeding on cellularity.* One-month-old male Wistar rats were fed either a high-lard diet (72% fat, 18% protein, 10% carbohydrate by calories) or a low-lard diet (9% fat, 18% protein, 73% carbohydrate by calories) for 1 or 10 months. These diets have been previously described (9). At the

[3] According to Michel (5), the depth of field (microns) is $= (10^3/7\ AM) + [\lambda\ (\mu m)/2\ A^2]$ with A = numerical aperture, M = total magnification, λ = light wavelength.

end of the respective feeding periods, the rats were killed and their epididymal fat pads were removed. Fat cell size and number were determined using the photomicrographic method. Data were analyzed by using Student's test.

Results. The results demonstrate that both methods used are readily reproducible and agree with each other. In duplicate experiments, the photomicrographic method showed a highly significant correlation between the duplicate runs ($r = 0.98$, $P < 0.001$), with only 1 out of 14 determinations showing more than 15% deviation from the identity line (Fig. 2A). Duplicate experiments, using the osmium fixation method, also showed a highly significant correlation between separate experimental runs ($r = 0.99$, $P < 0.001$) (Fig. 2B). A slightly greater variability was seen using the osmium method and may be related to a sampling artifact due to the small tissue samples taken for the osmium method. This is an important consideration since, in the usual application of the osmium fixation method, average cell size is indirectly determined. In comparing both rat and human samples, the cell sizes determined by the two methods were in excellent agreement ($r = 0.98$, $P < 0.001$). The regression line was not significantly different from the identity line (Fig. 2C). The average mean cell size and diameter for the 19 specimens were 0.349 μg of lipid/cell and 89.9 μm with the photomicrographic method, versus 0.353 μg of lipid/cell and 90.4 μm with the os-

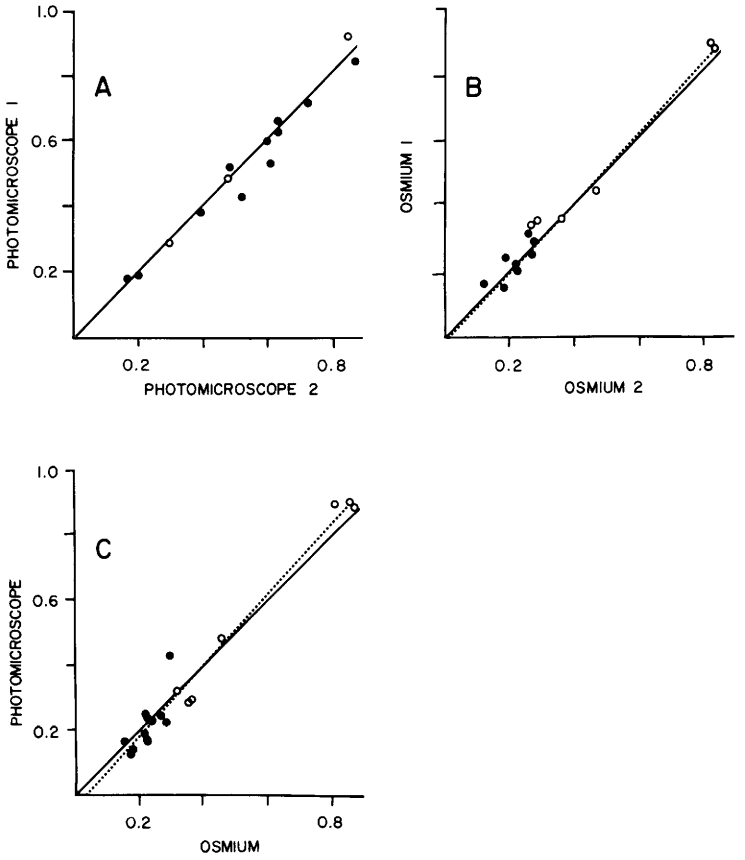

FIG. 2. Agreement between fat cell weight (micrograms of lipid per cell) determinations using: (A) the photomicroscope method in duplicate [$y = (1.01 \pm 0.058)\,x - 0.010 \pm 0.033$, $r = 0.98$, $n = 14$, $P < 0.001$]; (B) the osmium method in duplicate [$y = (1.04 \pm 0.047)\,x - 0.001 \pm 0.019$, $r = 0.99$, $n = 14$, $P < 0.001$]; (C) the photomicroscope method versus the osmium method [$y = (1.10 \pm 0.052)\,x - 0.042 \pm 0.022$, $r = 0.98$, $n = 19$, $P < 0.001$]. (——) Identity line; (+ +-) regression line; (●) rat adipose tissue; (○) human adipose tissue.

mium method. Furthermore, the agreement between methods was apparent over a wide range of cell size (0.15–0.90 μg of lipid/cell) (Fig. 2C). The comparison of cell size distribution also shows excellent agreement between these two methods, as may be seen in Figure 3, which shows that the frequency curves were nearly superimposable.

When Wistar rats were fed the high-fat diet for 1 month there was a significant enlargement of the epididymal fat pads as compared to the rats fed the low-fat diet (grams of lipid/2 pads = 2.4 ± 0.20 vs 1.5 ± 0.22, nine rats, $P < 0.01$). The fat cell size was increased by high-fat feeding (0.187 ± 0.0118 μg of lipid/cell vs 0.126 ± 0.0130, $P < 0.01$). There was no detectable change in the fat cell number ($12.5 \times 10^6 \pm 0.76 \times 10^6$ in two pads of fat fed rats vs $11.9 \times 10^6 \pm 0.88 \times 10^6$ in two pads of rats fed the low-fat diet, NS).

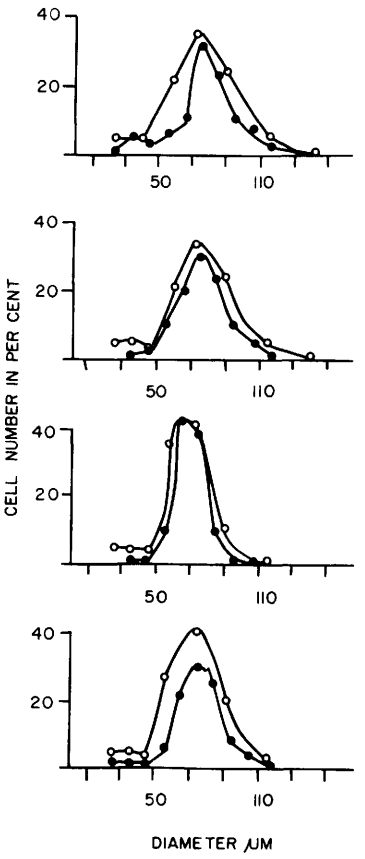

FIG. 3. Comparison of the distribution of diameter determined by sizing osmium-fixed rat fat cells with a Coulter counter (○) or by measuring the diameter of collagenase-isolated fat cells on photomicrographs (●). Curves were drawn by joining the midpoints of classes. Class limits were 1• μm with photomicroscope method and 10 or 15 μm with Coulter counter.

When Wistar rats were fed the high-fat diet for 10 months, considerable individual differences in weight gain were noted. In these experiments, both moderate (fat pad weight gain < 200% of control) and grossly obese rats were produced. In the moderately obese rats, only cell size enlargement was evident (Table I). In the grossly obese rats a statistically significant increase in cell number was noted after this 10-month feeding period (Table I). In the low fat-fed rat, the enlargement of the epididymal adipose tissue between 2 and 11 months was due solely to cell hypertrophy, as previously reported by Hirsch and Han (10).

Discussion. These results validate the photomicrographic method for determining fat cell size and number. Although many investigators have used collagenase digestion and manual sizing of isolated cells or osmium counting of cells, with the exception of the work of Hirsch and Gallian (1), the two methods have not been previously compared. However, in the comparative study reported by these authors (1), after isolation by collagenase, the fat cells were cooled for 1 hr at 4° and then fixed with trichloracetic acid and glutaraldehyde for an additional hour. This drastic treatment may explain "the great deal of cell breakage" reported by them. The method reported here, therefore, may be attractive to investigators for its cheapness and for its usefulness in determining relatively few samples. The measurement of fat cell diameter on photomicrographs provides a quick and permanent record of the sample which can be assessed at a future time. Thus, the method has the additional advantage of time flexibility and reduces the fat cell lysis that occurs when isolated fat cell samples must undergo both microscopic inspection and actual cell diameter measurements at one sitting. Since the agreement between the two methods is excellent in a wide range of cell sizes, it should be useful for both rat and human research.

The results of the high-fat feeding experiment not only demonstrate the applicability of this method, but are themselves interest-

TABLE I. LIPID CONTENT, FAT CELL WEIGHT, AND FAT CELL NUMBER IN EPIDIDYMAL ADIPOSE TISSUE OF 11-MONTH-OLD RATS FED EITHER A LOW- OR A HIGHT-FAT DIET FOR 10 MONTHS.

Diet	Lipid content (g/2 pads)	Fat cell weight (μg × 10^3 lipid/cell)	Fat cell (number × 10^{-7}/2 pads)
Low fat (5)[a]	5.5-9.30[b]	417-659	0.84-1.96
	7.18 ± 0.67[c]	510 ± 41	145 ± 0.20
High fat (9)	9.10-35.48	555-1150	1.42-4.15
	18.28 ± 2.66*	756 ± 63*	2.40 ± 0.28*
Moderately obese (4)	9.10-13.12	555-904	1.42-1.96
	11.81 ± 0.93*	706 ± 73**	1.69 ± 0.11 (NS)
Grossly obese (5)	17.33-35.48	555-1150	2.18-4.15
	23.45 ± 3.14***	806 ± 96*	2.96 ± 0.33***

[a] Number of animals.
[b] Range.
[c] Mean ± SE.
* Statistically different from the group fed the low-fat diet, $P < 0.025$.
** $P < 0.05$.
*** $P < 0.005$.

ing. The feeding of high-fat diets to lean rats has been shown to produce obesity in several, but not all strains of rodents. A hyperplastic effect on the epididymal fat pad has been reported by some authors (11, 12), but not by others (13). This work shows that in the short term (1 month), high-fat feeding in the Wistar rat alters fat cell size without any detectable effect on fat cell number. In the long term (10 months), the high-fat diet causes a substantial degree of weight variability. However, those rats which store large amounts of fat have large cells and show a significant increase in cell number compared to low fat-fed rats. In this long-term experiment, the same high-fat diet treatment induced either a moderate obesity with hypertrophic enlargement or a more severe obesity with both hypertrophic and hyperplastic changes.

It is not possible to be certain whether the apparent fat cell hyperplasia is a result of new cell formation or of fat-filling of existing small, undetectable fat cells. Recently, Johnson *et al.* (14) have reported that, when Osborne Mendel rats were fed a high-fat diet (55% by weight) for 9 weeks, apparent fat cell hyperplasia of the retroperitoneal depot occurs. They reported that feeding for a shorter period, i.e., 3 weeks, increased only cell size. It is becoming increasingly clear that the time that treatment begins, the length of feeding, strain of rats, individual caloric consumption and activity, and the depot examined may all be important variables to consider when reporting the results of dietary fat alterations on adipose tissue morphology.

Summary. Rat and human isolated adipocyte sizes were determined by both the measurement of their diameter on photomicrographs and by electronic counting of osmium-fixed cells. The average cell size was 0.348 and 0.353 μg of lipid/cell, respectively. The agreement between the two methods was excellent over a wide range of cell sizes (0.15-0.09 μg of lipid/cell). The photomicrographic method was applied to a morphological study of epididymal adipose tissue in Wistar rats fed a high-fat diet for either 1 or 10 months. The enlargement of the adipose depot induced by 1 month of the high-fat diet was achieved by an increase of fat cell size only. Ten months of high-fat feeding induced either a moderate obesity with only hypertrophy of the adipocytes or a severe obesity with apparent hypertrophic-hyperplastic changes.

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Received April 22, 1977. P.S.E.B.M. 1977, Vol. 156.