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A simple method to predict cellular density in adipocyte metabolic incubations

JB Fine and M DiGirolamo

Department of Medicine, Emory University School of Medicine, Atlanta, GA 30322, USA

OBJECTIVES: The density of isolated adipocyte suspensions, namely the cellular concentration, influences metabolic results when lipolysis and the pattern of glucose metabolism are studied. It is often difficult to obtain reproducible adipocyte concentrations from experiment to experiment, and investigators usually measure the cell concentration at the end rather than at the initiation of metabolic incubations.
METHOD: A simple and rapid method to obtain reliable and predictable adipocyte concentration *prior* to metabolic incubations is described and validated. The method is based on determination of lipocrit, mean adipocyte diameter (by optical sizing), and calculation of volume, in aliquots of isolated adipocyte suspensions.
MAIN OUTCOME MEASURES: Lipocrit, mean adipocyte volume, predicted and observed adipocyte number in isolated cell suspensions.
RESULTS: In 15 experiments, adipocyte concentration was accurately predicted within 12–18% of actual concentration. This is in contrast to the four- or five-fold differences usually encountered in a series of experiments.
CONCLUSION: One can rapidly predict the number of adipocytes present in a given cell suspension with the proposed method, and then correct it to a desired adipocyte concentration at the *beginning* of metabolic incubations. This method will help to eliminate the confounding effects of variable cell concentrations in *in vitro* metabolic experiments with isolated adipocytes.

Keywords: adipocyte suspensions; adipocyte number and volume; lipocrit; cell density

Introduction

Preparations of isolated adipocytes are commonly used to determine cellular metabolic characteristics and responses to hormones.[1–6] It is known, however, that marked variability of metabolic results exists both between laboratories and within the same laboratory from experiment to experiment. The reasons for this variability are not entirely clear.

An important factor that has been recognized recently is the density of isolated adipocytes in a given incubation volume. Adipocyte concentration has been shown to affect significantly the rates of cellular metabolism. Schwabe *et al*[7,8] have reported that increases in adipocyte concentration result in lower rates of lipolysis per cell. More recently, our laboratory[9] has reported that increments in fat cell density increase the relative rates of glucose metabolism to CO_2 and fatty acids, and lower the relative rates of glucose conversion to glyceride-glycerol, lactate, and pyruvate. Specifically, when adipocyte concentration was systematically changed from 168,000 to 635,000 cells/ml incubation, the relative conversion of glucose to CO_2 and fatty acids increased from 20 to 40% and from 9 to 24%, respectively. Conversely, the relative conversion of glucose to glycerol, lactate, and pyruvate decreased from 34 to 20%, from 20 to 11%, and from 14 to 5%, respectively. Furthermore, the response of adipocytes to insulin was greater at higher cell density than at lower density.[9] These findings demonstrate convincingly the importance of adipocyte concentration in metabolic incubations and point to the need for a relatively simple method to obtain a desired and consistently reproducible cell concentration *prior* to metabolic incubations.

In this study, we proposed (and validated) a rapid and simple method to estimate adipocyte concentration that is based on an easily obtained lipocrit (analogous to a haematocrit) in an isolated cell preparation, and on the determination of mean adipocyte diameter and volume by an optical sizing method.[10] With these procedures, and relatively simple calculations, one can predict the number of adipocytes in a given suspension and, eventually, correct it to the desired number in a given incubation volume. In this fashion, a series of experiments can be carried out at a desired and consistent adipocyte concentration, and cell density effects on known and unknown cellular metabolic parameters can be controlled.

Correspondence: Dr M DiGirolamo, Professor of Medicine and Physiology, Emory University School of Medicine, Geriatrics/WMB, 1639 Pierce Drive, N.E., Atlanta, GA 30322, USA.
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Methods

Animals

Male Wistar or Sprague-Dawley rats, age 6 weeks to 10 months, purchased from Charles River Laboratories, were housed in constant conditions, on a 12:12 light/dark schedule, at 23°C, and offered Purina Laboratory Chow (#5001) and water *ad libitum*. The animals were killed by CO_2 asphyxiation and the two epididymal fat pads were excised. Following removal of visible vascular tissue, the pads were weighed to the nearest mg.

Preparation of isolated adipocytes

The adipose tissue was minced with scissors and then incubated in Krebs-Ringer bicarbonate medium (KRB) with 4% bovine serum albumin (Sigma Co.) in the presence of Type I Collagenase (Worthington Biochemicals) at 1.5 mg/ml of KRB-albumin medium (pH 7.4). At the end of 45 min incubation at 37°C in a Dubnoff metabolic shaker (80 cycles/min), the digested tissue was passed through a 250 µm nylon screen. The resulting cell suspension was washed × 3, in KRB-4% BSA medium without collagenase, by removal of the infranatant below the cells that floated above the medium by gravity or gentle centrifugation (1000 × *g* for 1 min). On the third and final wash, the visible cell layer was monitored and enough fresh medium was added to make the cell layer approximately 20–25% of total volume.

Determination of adipocyte size

Aliquots of the evenly suspended isolated adipocytes were removed for optical sizing of cell diameter and for determination of lipid (triacylglycerol) content (see below).

Optical sizing was done after allowing the adipocyte suspension to stain in 1% methylene blue at 37°C for 3–5 min. The cells were then placed on a siliconized microscope slide inside a circular well made by silicone vacuum grease (Dow Corning Co.) squeezed through a plastic syringe. A microscope cover slip was placed above the silicone well, and gently pushed downward until the cells were in contact with the cover slip.

A Zeiss microscope, equipped with a micrometer grid, was used to measure the transverse diameter of 200–300 isolated adipocytes. Details of cell isolation, sizing and calculation of mean adipocyte diameter and volume were reported previously.[10]

Determination of lipocrit in adipocyte suspension

Six aliquots of evenly suspended isolated adipocytes were aspirated in haematocrit capillaries (Scientific Products), siliconized with Sigmacote (Sigma Co.); one end was sealed with S/P Sure-seal tube sealant (Baxter Scientific Products). The capillaries were centrifuged in a Readacrit Centrifuge (Clay Adams/Becton-Dickinson Co.). At the end of one minute centrifugation at approximately 8000 × *g*, the capillaries were removed and the lipocrit (namely the column occupied by the packed adipocytes divided by total volume) was assessed with the help of a Spirocrit Microhematocrit Capillary tube reader (from Oxford Labware Co.).

The means of six replicate lipocrit values, and the standard deviations (s.d.) were calculated for each experiment. Using these values, the coefficients of variation (CV = s.d./mean) were assessed.

Prediction of adipocyte concentration and validation by direct measurement of adipocyte number in suspension

The lipocrit (in six repeated observations) can be obtained in 3–5 min. The calculated mean lipocrit value reflects the packed volume occupied by adipocytes floating above the aqueous medium.

The mean adipocyte volume can be obtained, in parallel, by optical sizing of the diameter of 200–300 cells. With some experience, sizing and calculation of mean (± s.d.) adipocyte diameter and volume[10,11] can be completed in 5–7 min.

By dividing the mean adipocyte volume into the volume occupied by packed adipocytes (lipocrit), it is possible to calculate the predicted number of cells present per ml of initial cell suspension. The original cell concentration can be diluted to the desired concentration by adding the appropriate volume of medium.

An example of the steps required to predict adipocyte concentration and of the steps needed to reach the desired concentration is shown below and in Figure 1.

(a) µl volume occupied by packed adipocytes (lipocrit × 10)/mean adipocyte volume (pl) = adipocyte number/ml in initial suspension.
(b) (Adipocyte number/ml in initial suspension)/(desired adipocyte number/ml in final suspension) = dilution factor.

Measurement of lipid (triacylglycerol) content in aliquots of the initial and final cell suspension was taken to validate the predicted final cell concentration. Specifically, three aliquots of the cell suspension were extracted in Dole's mixture.[12] The mean total lipid (triacylglycerol) content of the three aliquots was then assessed gravimetrically after samples were dried overnight in a vacuum oven. The number of adipocytes in the suspension could be calculated by dividing total lipid/ml by the mean adipocyte lipid (mean cell volume × density 0.915).[13] The lipid extraction and calculation of actual cell number requires a minimum of 12–24 h. Thus, this procedure cannot be performed prior to metabolic experiments. It does, however, provide an *a posteriori* measure of accuracy for the prediction of cell density.

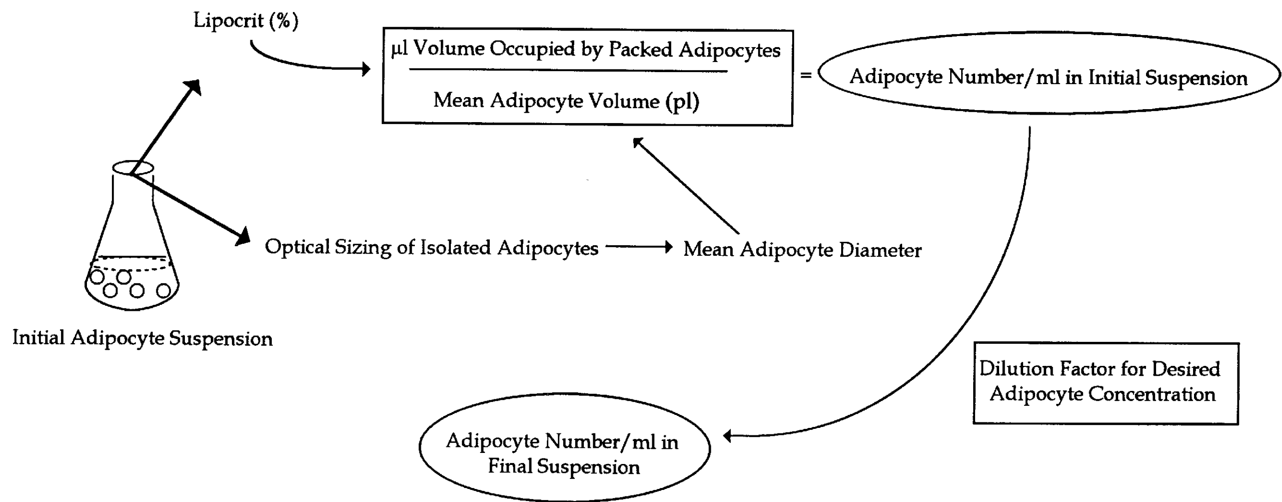

Figure 1 Schematic representation of the steps involved in the prediction of adipocyte concentration and its correction to a desired cell concentration *prior to* metabolic incubations.

Statistical evaluation of the data
Means, standard deviations and standard errors of the mean were calculated in the usual way. The significance of the differences between the group means (predicted and observed values), and between the correlation coefficient and zero was estimated by the Student's t-test (Jandel Sigmastat Statistical Software, version 2.0). Values for $P < 0.05$ were taken to indicate significance.

Results

Animals and preparation of isolated adipocytes
The 15 animals used varied in: age, from 6 weeks to 10 months; body weight, from 150 g to 789 g; epididymal fat pad weights, from 0.625 g to 19.22 g; and mean adipocyte size in the epididymal fat pads, from 63 pl to 570 pl (see Table 1).

Accuracy of lipocrit determination
The accuracy and reproducibility of the lipocrit determination was assessed by six repeated observations in the adipocyte suspensions.

The coefficient of variation (s.d./mean) of each set of lipocrits, averaged for the 15 experiments was 0.045, thus indicating a good measure of precision in this critical determination. We recommend a minimum of 5–6 repeated lipocrit determinations for this measure, in view of the difficulties related to cell fragility and evenness of the cell suspension. To improve the reproducibility of the sampling of aliquots from the same cell suspension, we recommend both the use of Erlenmeyer flasks, 100–125 ml, and a

Table 1 Summary of 15 separate experiments

Experiment no.	*Mean adipocyte volume (pl)*	*Lipocrit (%)*	*Initial cell suspension relative difference (%)*	*Final cell suspension relative difference (%)*
1	63	6.75	10.52	30.92
2	66	16.5	39.51	42.26
3	91	12.2	2.71	32.83
4	93	10.83	4.96	25.4
5	113	24	11.27	11.99
6	119	7.08	−5.64	−6.2
7	120	10	36.62	42.65
8	156	15.5	5.78	no dilution
9	232	17	13.4	22.22
10	250	20.3	13.67	8.91
11	281	22.2	17.51	18.04
12	287	19.6	8.38	1.71
13	310	16.83	3.51	5.92
14	363	20.6	10.97	19.91
15	570	20.83	6.91	−9.42
			mean = 12.01 ± 3.09	mean = 17.65 ± 4.40

Data for each experiment are ranked in ascending order on the basis of mean adipocyte volume. Mean adipocyte volume was obtained by the optical sizing method. The lipocrit (%) values are the averages of six replicate determinations in each experiment. The relative difference (in % ± s.e.m.) was obtained by comparing the predicted adipocyte number values for the initial or final cell suspension (adipocyte number/ml) to the appropriate observed values. See also Figure 2 and text for details.

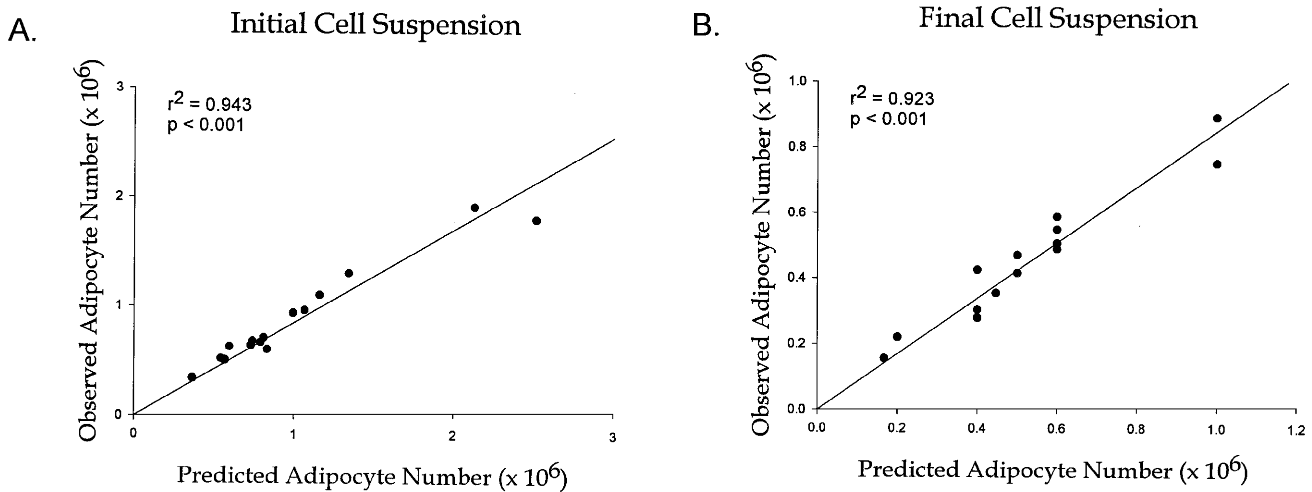

Figure 2 This figure shows the relation between predicted and measured adipocyte number in the initial cell suspension (panel A) and in the final suspension (panel B), in 15 experiments with isolated adipocytes from epididymal adipose tissue of rats varying in age and degree of adiposity (see Table 1). Predicted values were obtained from six repeated lipocrit determinations, optical sizing of cell diameter and calculation of mean adipocyte volume. Observed values were determined from triplicate measurements of cell lipid (triacylglycerol) and calculation of adipocyte number by determining mean cell lipid content (cell volume × 0.915 density). See text for details.

gentle, circular arm and wrist motion (prior to sampling) to produce an even fat cell suspension and prevent adipocytes from floating to the top of the flask during sampling.

Prediction of adipocyte concentration by lipocrit and estimation of cell volume in initial cell suspension and in the final diluted suspension

By combining lipocrit values with the mean adipocyte volume, it is possible to predict cell density in the initial cell suspension. This step is rapid and reproducible. Note that lipid (triacylglycerol) determination *is not* necessary for the cell density estimate.

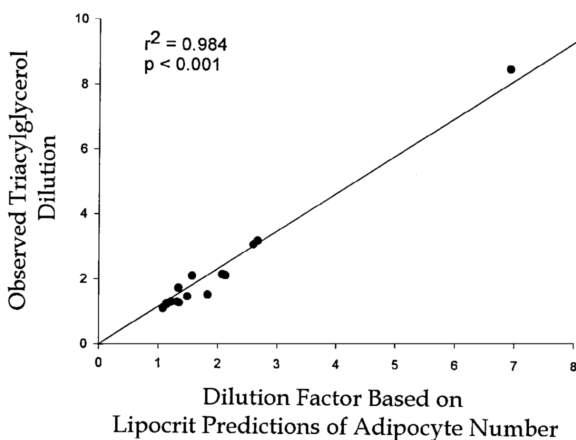

Figure 3 This figure shows the relation between the dilution factor used to reach desired adipocyte concentration on the basis of predicted adipocyte number, and the equivalent dilution of the triacylglycerol content of aliquots of the initial and final cell suspensions. The tight correlation ($r^2 = 0.984$) is an indication of the procedural precision encountered. See text for details.

When the adipocyte concentration derived from triacylglycerol values was compared to the prediction of cell number obtained by lipocrit values in the initial cell suspension, predicted values were not significantly different from values obtained by triacylglycerol count ($P = 0.43$); on average, the predicted values exceeded the measured adipocyte concentration by 12%. Figure 2 shows the relation between the predicted values for adipocyte number (based on lipocrit and adipocyte volume) and the observed values (based on measurement of triacylglycerol content of aliquots of the cell suspension). Panel A shows the relation for the initial adipocyte suspension ($r^2 = 0.943$, $P < 0.001$), and panel B, that for the final cell suspension ($r^2 = 0.923$, $P < 0.001$).

Table 1 also shows that the predicted final cell suspension averaged ~ 18% in excess of the observed values. However, this small difference between the predicted and observed values was not statistically significant ($P = 0.36$). The relation between the cell dilution factor employed and the observed lipid (triacylglycerol) dilution is shown in Figure 2. We also have noticed that visual estimates of the adipocyte layer (as % of total) at wash #3 are about double of what the lipocrit values show.

Discussion

We have described a simple, accurate and rapid method to obtain a desired and consistently reproduced adipocyte concentration for metabolic incubations. Validation of the various steps involved and of the final estimate of cell concentrations shows that it is now possible to narrow the desired concentration to about 12–18% of the expected one under variable conditions of adipocyte size and concentrations. This

is in sharp contrast to various estimates of cell density found to vary up to 5-fold from experiment to experiment even in laboratories, such as ours, that usually attempt to obtain a constant cell density.

We have used two strains of rats and carried out observations with epididymal adipocytes varying markedly in volume from 63–570 pl. In view of the uniformity of populations of isolated adipocytes from many animal species,[10] this method can be applied to any species or adipose depot from which isolated adipocyte suspensions can be obtained.

About 8–10 min at the end of the preparation of isolated adipocytes and *prior* to the initiation of the metabolic incubation is all that is required to obtain a consistently reproducible adipocyte concentration. Some investigators have alluded to the use of lipocrit to obtain a desired cell density, but no details were given of methodology, reliability, or validation in their papers (for example, see Reference 14). It is possible that, at times, the visual adipocyte layer was used, but we have found that to be unpredictable and imprecise in adipocyte populations of variable cell volume.

In this work, in spite of the fact that prediction of the number of cells in suspension was very accurate (within 12–18% of measured values), the predicted values appeared to be slightly in excess of the determined number. A possible explanation may reside in the water content of the adipocytes themselves (usually 4–6% of cell volume in cells < 100 pl and 1–2% of cell volume in cells > 100 pl), and also in the water trapped between the fat cells in the preparation of the lipocrit, found to be about 0.2% of the aqueous medium.[15] Although small, this water content can provide a slight overestimate of the lipocrit value and thus be responsible for the observed discrepancy.

In view of the previous findings of the importance of *in vitro* adipocyte concentration in modulating adipocyte lipolytic activity,[7] patterns of glucose metabolism[9] and insulin response, we believe that the proposed methodological improvement can contribute to a more precise study of the effects of variable cell density on metabolic performance by isolated adipocytes.

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