Quantitative analysis of rat adipose tissue cell recovery, and non-fat cell volume, in primary cell cultures

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Background. White adipose tissue (WAT) is a complex, disperse, multifunctional organ which contains adipocytes, and a large proportion of fat, but also other cell types, active in defence, regeneration and signalling functions. Studies with adipocytes often require their isolation from WAT breaking up the matrix collagen fibres, but primary cultures of these cells could not be easily correlated to intact WAT, since often recovery and viability are unknown. *Experimental design*. Epididymal WAT of 4-6 young adult rats was used to isolate adipocytes with collagenase. Careful recording of lipid content of tissue, and all fraction volumes and weights, allowed us to trace the amount of initial WAT fat remaining in the cell preparation. Functionality was estimated by incubation with glucose and measurement of lactate production. Non-adipocyte cells were also recovered and their sizes (and those of adipocytes) were also measured. The presence of non-nucleated cells (erythrocytes) was also estimated. *Results.* Cell numbers and sizes were correlated from all fractions to intact WAT. Tracing the lipid content, the recovery of adipocytes in the final, metabolically active, preparation was in the range of 70-75%. Adipocytes were 7%, erythrocytes 68% and other stromal (nucleated cells) 24% of total WAT cells. However, their overall volumes were, 91%, 0.05%, and 0.2% of WAT. Non-fat volume of adipocytes was 2.5% of WAT. *Conclusions.* The methodology presented here allows for a direct quantitative reference to the original tissue of studies using isolated cells. We have found, also, that the "live cell mass" of adipose tissue is very small (about 25 µL/g for adipocytes and 2 µL/g stromal, plus about 1 µL/g blood). This fact, translates into an extremely high (with respect to the actual "live cytoplasm" size) metabolic activity, which make WAT an even more significant agent in the control of energy metabolism.

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Introduction

White adipose tissue (WAT), which has been defined as the adipose organ (Cinti 2001), is dispersed in a large number of locations, in which its basic energy storage activity is complemented by many other physiological functions (Alemany & Fernández-López 2006). In any case, its main acknowledged role is to contribute to the defense of energy homoeostasis, helping to control glucose (Sabater et al. 2014), lipid (Deschênes et al. 2003; Wang et al. 2016), and amino acid (Arriarán et al. 2015a) metabolism overall. It is responsible for an important share of the control of whole body energy availability (Choe et al. 2016; Hall et al. 2009), and acts as a platform for the immune system, being actively implicated in processes of protection and repair (Dixit 2008; Parker & Katz 2006). The complex (and varying) mixture of cell types in WAT sites largely determines and modulates these functions as part of its protean adaptability (Oishi & Manabe 2016; Vielma et al. 2013).

Most of WAT volume is taken up by a relatively small number of large cells, the mature adipocytes, which are generally considered the genuine cells of this tissue and thus the main target for the fight against obesity (Nawrocki & Scherer 2005). However, most of the adipocyte volume is filled by (triacylglycerol) energy reserves (Kotronen et al. 2010). This can be extended, obviously in similar proportions, (often higher than 80%) to the WAT/ adipose organ taken as a whole. This is a variable but significant share of total body weight (5-50%) in humans and most animal phyla. The rest of WAT cells are loosely called stromal, despite most of them not being actually connective tissue cells (da Silva Meirelles et al. 2015). In this text, we will use the general term "stromal cell" to refer to all WAT cells different from fat-laden adipocytes.

The stromal fraction of WAT is made up of immune system, stem, blood, endothelial, true stromal and other types of cells, with relevant functions in the maintenance of adipocyte energy homoeostasis (Sadie van Gijsen et al. 2012), defense (Hill et al. 2014), regeneration (Domergue et al. 2016), differentiation (Gimble et al. 2011; Mitterberger et al. 2014) and others (Sumi et al. 2007; Takahara et al. 2014). Many of these functions become critical under conditions of inflammation (Lee 2013), changing the cell composition and overall WAT metabolism (Cignarelli et al. 2012; Lolmède et al. 2011). Adipocytes, despite their small numbers (but huge volume due to their fat stores), have been intensely studied as "representative" of WAT (Leonhardt et al. 1978). To study their metabolic or regulatory capabilities, the cells are isolated from WAT masses and studied using primary (Garvey et al. 1987) or immortalized (Tordjman et al. 1990) cell cultures. The information obtained is often taken as directly representative of WAT *in vivo,* in spite of the large number of factors that are known to rebut this excessively simplistic approach (O'Brien et al. 1996), including the ordeal of cell isolation (Thompson et al. 2012).

When dealing with WAT, the data obtained from most experiments is deeply conditioned by the methodology used, i.e. isolated cells, tissue pieces or slices, or *in vivo* functional analyses. Seldom can we obtain quantitative data which could be referred to the live tissue. Comparison of different locations, individuals, metabolic or pathologic conditions is severely hampered by the size of fat depots (Cinti 2001; Wronska & Kmiec 2012), the varying proportion of adipocyte/stromal cells (in fact, only when the latter are actually taken into account (Pasarica et al. 2009)) and the blood flow/oxygen and substrates' availability (Mjös & Akre 1971). Quantification of adipocyte recovery from whole tissue samples, and the analysis of the proportion of "live" cell space in the tissue are necessary steps for direct comparison of data from different sources. Unfortunately, cell number is dependent on the method of quantification used, and is logically affected by cell volume. The proportion of fat in the tissue and cells also proportionally "reduces" the live-cell mass. This is further compounded by the direct estimation of cells via DNA analysis since (at least in mammals) red blood cells are not computed, and a small hematopoietic cell (Luche et al. 2015) macrophage or lymphocyte (Sell & Eckel 2010) count as a cell the same than an adipocyte with a volume about 10^5-fold larger.

Referring cell or tissue experimental data to protein content may be a fair index for comparison, but the large presence (also deeply varying depending on location (Alkhouli et al. 2013)) of extracellular fibrous proteins, such as collagen (Liu et al. 2016) also modifies the quantitative evaluation of the metabolically active fraction of the tissue; this fraction is also deeply affected by obesity and inflammation (Li et al. 2010).

In the present study, we have devised a method for the estimation of actual adipocyte recovery of viable adipocytes with respect to WAT mass based on the unique presence of large amounts of fat in them. We have also intended to present an estimation of the size of the metabolically active WAT cell mass with respect to the mass/ volume of the tissue. We used, as reference, the epididymal WAT fat pads of non-obese healthy young adult rats (to limit the known effects of inflammation on WAT cell profile). This location is considered to be one of the less metabolically active (Arriarán et al. 2015b), and is widely used for "representative" WAT adipocyte function for its size, easy dissection and absence of contamination by neighboring tissues.

Materials and Methods

Rats and housing conditions

All animal handling procedures and the experimental setup were in accordance with the animal handling guidelines of the corresponding European and Catalan Authorities. The

Committee on Animal Experimentation of the University of Barcelona specifically authorized the procedures used in the present study.

Male Wistar rats (Harlan Laboratory Models, Sant Feliu de Codines, Spain), 18-week old, weighing 435±32 g, were used after a 2-week acclimation period in a controlled environment. The animals were kept in two-rat cages with wood shards as bedding material, at 21-22ºC, and 50-60% relative humidity; lights were on from 08:00 to 20:00. They had unrestricted access to water and standard maintenance rat chow (Harlan #2014).

Isolation of adipocytes

The rats were killed, under isoflurane anesthesia, at the beginning of a light cycle, by exsanguination from the exposed aorta, using dry-heparinized syringes. The rats were rapidly dissected, taking samples of epididymal WAT, used immediately for adipocyte isolation. This procedure followed, essentially that described by Rodbell (Rodbell 1964). In short, tissue samples were weighed, immersed in the digestion medium described below, and cut in small pieces with scissors. Samples were incubated, at 37ºC in a shaking bath for 60 min, with 2.5 volumes of Krebs-Henseleit buffer pH 7.4, containing 5 mM glucose, 0.1 μM adenosine (Sigma-Aldrich, St Louis MO USA) (Honnor et al. 1985), and 10 g/L lipid-free bovine serum albumin (Merck-Millipore, Billerica, MA USA). This was complemented with 3.5 mkat/L collagenase (LS004196, type I, from Worthington Biomedical, Lakewood NJ USA). At the end of the digestion process, the suspensions were gently sieved using a double layer of nylon mesh hose, which retained vessel fragments and (eventually) undigested tissue pieces. The smooth crude suspension of isolated cells was left standing for 5 minutes on stoppered plastic syringes, held vertically. The adipocytes floated to form a defined upper layer. Then, the lower aqueous fraction was slowly drained off, capping again the syringe to retain the adipocytes. The cells were washed this way three times, using 2.5 volumes of the buffer. Before re-suspending the cells in it, the buffer was subjected to 30 s vortexing, to allow for equilibration with air oxygen. The final supernatant fraction contained intact adipocytes and a thin layer of free fat from broken cells. After the final washing, aliquots of the cells' fraction were taken for incubation. The samples were extracted from the central part of the fraction, trying not to disturb the thin floating fat layer. The cells were manipulated and maintained at room temperature for a time as short as possible, and used immediately after the final washing.

Stromal cell space in the isolated cell suspension, used to relate their numbers and volumes to initial tissue weight, was considered the sum of the lower phase of adipocyte separation in the syringes, plus the volume of the adipocyte phase to which the volume of adipocytes (calculated from cell numbers and volumes) was subtracted. Obviously, the first separation of adipocytes and stromal cells left a high number of the latter mixed with adipocytes. The three successive washings

resulted in the presence (calculated) of, at most, 0.1 % of the initial stromal cells in the final washed adipocyte fraction (down from an initial 7.3 %). This assumption does not take into account stromal cells bound, retained or attached to the large adipocytes.

Estimation of the efficiency of adipocyte extraction

The only cells in WAT containing significant amounts of fat in intracellular depots are the adipocytes (i.e. sufficient to generate enough floatability to allow their separation from the rest of cells just standing –i.e. at 1 x *g*— for a few minutes). We used this differential fat content to establish an approximate estimation of the efficiency of the digestion-extraction procedure for adipocyte isolation described above, simply by estimating the recovery of fat from the intact tissue to a preparation containing only viable functional cells.

A sample of just dissected WAT was divided in two parts, one was processed to obtain washed adipocytes as described above, and the other was divided in several aliquots, used to measure the water (dry weight after 24 h at 90 ºC) and lipid content. To measure lipids, fragments of about 300 mg of intact tissue were weighed and extracted with trichloromethane: methanol (2:1 v/v) (Folch et al. 1957). The resulting values were used to establish the proportion of lipids in the intact tissue. Using this method as originally described, most of membrane lipids were not extracted (Eder et al. 1993; Rose & Oklander 1965), but the recovery of WAT-vacuole lipids (i.e. fat, essentially triacylglycerols) was quantitative. The weights of the lipids present in the fat layer on top of the cells' suspension (washed and essentially free of stromal cells, as explained above), and those of stromal cells' fraction and extraction debris were measured. The weight of the recovered adipocyte fraction and their water and lipid content were also estimated, thus obtaining the total weight of lipid present in the isolated adipocytes.

The density of WAT was estimated using tightly capped tubes, which were weighed both dry and completely full of deionized water at 20 ºC. The net weight of water was used to calculate the volume of the tube. The process was repeated including weighed 300-500 mg pieces of intact WAT in the tubes and completely filling them with water (nevertheless, no different values were obtained using pieces of 200 to 1000 mg). The difference in weight of the tubes with and without WAT samples allowed us to calculate the volume of the samples; their density was estimated from the volume and weight. Other samples of WAT were used to extract its lipid as described above. The density of the extracted lipid was estimated using the same procedure using cold-solidified fat samples.

The weight of lipid extracted from the adipocyte preparation was compared with the initial weight and the actual proportion of lipid present in the intact tissue, after discounting the weight of debris eliminated during the process of extraction. Lipid in the stromal cell fraction was negligible, statistically not different from zero.

Measurement of isolated cell parameters

A known volume of the suspension of adipocytes was introduced in a Neubauer chamber (#717810 Neubauer improved bright line, Brand Gmbh, Wertheim Germany). Using an inverted microscope, four fields (following a pre-established selection pattern) were photographed at low power (Figure 1). Four samples of each adipocyte suspension were inspected, taking 16 photographs from each. Cells were counted, and their diameters analyzed (under the conditions used, all cells adopted a spheroid form), using the *FIJI ImageJ* software (http://imagej.nih.gov/ij/), following a simple procedure (Baviskar 2011). The data were computed (range, mean and SD for diameter, cell volume and number, including their combined volume). In this experiment, the final range of counted cells (mean, SD) was 92 ± 18 µm in diameter (when assuming the form of a sphere), i.e. 472 ± 285 pL in volume. Figure 2 shows a representative example of the range of cell sizes obtained using this procedure on epididymal WAT.

Non-nucleated cells (essentially red blood cells: RBCs) were identified by their smaller size (in the fL range) using the Scepter 2.0 cell counter (EDM Millipore Corp, Billerica, MA USA) hand-held cell sizer. Total stromal cells, (i.e. including RBCs) were analyzed for each sample using two different cell-range tips for the Scepter: Sensor 40, for 3-18 µm particles' size (PHCC40050, Merck Millipore, Darmstadt, Germany) and Sensor 60, for 6-36 µm particles' size (PHCC60050, Merck MIllipore). The data for both ends of the superimposed size graphs were taken as final values, and those in the overlapping zone were used taking in both series of data against diameter. After the data were arranged, the measured volumes were plotted and the data were analyzed statistically.

Using stromal cell fraction samples from all rats tested, a cytometric flow analysis (Figure 3) was performed to distinguish the proportion of small non-nucleated cells (i.e. red blood cells) from those nucleated and either dead or viable. The analyses were done using a FacsAria I SORP sorter (Beckton-Dickinson, San Jose CA USA). The cells were stained with propidium iodide (Sigma-Aldrich) and Syto-13 Life Technology, Thermo-Fisher Scientific, Waltham MA USA) used to estimate the proportion of non-nucleated red blood cells in the samples as a percentage of total stromal cells. We used this value to estimate the presence of blood cells in the whole tissue and stromal cell counts, incorporating these data in the calculations.

Cell viability

We analyzed the functionality of the cells checking their metabolic integrity along a 2-day incubation study. We used 12-well plates (#CLS3513 Costar, Sigma-Aldrich) filled with 1.7 ml of DMEM (#11966-DMEM-no glucose; Gibco, Thermo-Fisher Scientific, Waltham MA USA), supplemented with 30 mL/L fetal bovine serum (FBS, Gibco). The medium (Romero et al. 2015) also contained 25 mM hepes (Sigma-Aldrich), 2mM glutamine (Lonza Biowhittaker, Radnor, PA

USA), 1 mM pyruvate (Gibco), 30 mg/mL delipidated bovine serum albumin (Millipore Calbiochem, MA USA), 100 U/mL penicillin and 100 mg/L streptomycin (Gibco). Adenosine (Sigma-Aldrich) 100 nM was also added to help maintain the integrity of the cells. D-glucose (7 mM) was added as substrate. Each well received 400 µL of the adipocyte suspension, thus completing a final volume of 2.1 mL. Under these conditions, the cells floated freely (as spheres) and tended to accumulate on the surface of the well. The cells were incubated at 37°C in an incubation chamber ventilated with air supplemented with 5% CO_2, which gave a theoretical pO_2 of 20 kPa (i.e. 0.2 mM of dissolved O_2) (Romero et al. 2015). The calculated pCO_2 was in the range of 5 kPa, corresponding to 1.7 mM of dissolved CO_2. The cells were incubated for 24 h or 48 h without any further intervention. At the end of the experiment, a sample of the well contents was used to determine the number of cells. Then, the cells were harvested and the medium was extracted and frozen.

The incubation medium was used for the estimation of glucose, using a glucose oxidase-peroxidase kit (#11504, Biosystems, Barcelona Spain) to which we added 740 nkat/mL mutarrotase (porcine kidney, 136A5000, Calzyme, St Louis, MO USA) (Oliva et al. 2015). Lactate was measured with kit 1001330 (Spinreact, Sant Esteve d'en Bas, Spain).

Calculations

A critical factor in the development of this procedure was to keep track of all weights/ volumes and incorporate into the calculations all aliquots extracted for testing (i.e. glucose or lactate levels). All data were introduced in a spreadsheet in which the volumes were justified with a (pipetting) error of ± 3%. When possible, or when no other avenue was available, volumes were estimated from differential weights and the application of the densities calculated as described above.

The calculations used to determine the cell parameters, adipocyte recovery and WAT cell distribution are described in the Tables, presenting the original experimental data along with the derived or calculated data, as well as the formulas used for their estimation.

Statistical analyses were carried out using the Prism 5 Program (Graphpad Software Inc., La Jolla, CA USA).

Results

Analysis of the recovery of adipocytes from intact epididymal WAT

Table 1 shows the main experimental data for the quantitative analysis of free isolated adipocyte yield from just-dissected epididymal WAT. Both weight, water and fat content, as

expected, showed little variation. The suspension obtained after collagenase digestion was estimated by weight, as were the floating fat layer and the debris retained in the nylon mesh. The number, and mean volume of intact adipocytes was also fairly uniform (since the measurement implied hundreds of individual counts, we decided to show the SD values instead of the extremely low corresponding SEM to present a better image of the cell diversity). The number of free (i.e. unattached to adipocytes) stromal cells was 17-fold higher than that of adipocytes, but almost 3/4^{ths} of them were just red blood cells. All stromal cells had cell volumes in the range of 10^{-4} of those of adipocytes. The volumes of all stromal cells, including erythrocytes were measured after separation via high-speed centrifugation, which may have altered their original shape and volume, a treatment that the large adipocytes could not endure.

All tables contain a first column, labeled #, in which a letter and number are given to each row (or datum). These references are later used, in Tables 2 to 6 to present the origin of the data and the calculations done using the experimental data.

Table 2 presents the calculations (largely based on the data in Table 1) used to determine the recovery of viable isolated adipocytes from the intact tissue sample. Since all experimental data referred to weight (its measurement was several-fold more precise than volumetric measurements, especially those implying solids –such as cells— in suspension and mixed-phase systems) the main column of data is that indicated by weights, and have been referred to mg in 1 g of initial tissue. These values were converted to volumes using the densities experimentally measured for fat and tissue shown in Table 1. The third column shows the origin of the data and the calculations used to obtain the corresponding values.

The detailed calculations of the efficiency of adipocyte recovery can be seen on Tables 1 and 2: in the calculations, we used both weights and volumes (using their equivalences *via* experimentally determined densities of both intact WAT and extracted WAT fat). We assumed that practically all WAT fat was present only in adipocytes, since membrane lipids were not extracted with the procedure used, and the presence of fat in stromal cells went undetected and, in any case, could not represent a significant amount of material given the combined volume of these cells and their density. Consequently, all the fat present in the final intact adipocyte preparation should correspond to that of adipocytes, since free fat was measured and removed, and there were no other fat-carrying cells in the system. Thus, we could equate the losses of fat (with respect to intact tissue) with losses of adipocytes. These losses were found to be significant, and the manipulation of the cells resulted in additional cells breakup. Under the conditions described, the collagenase incubation and extrusion through the nylon mesh resulted in a loss of about 24% of the cells (in fact, losses of fat), and the washings of the isolated cells added an additional loss in

the range of 3 %, which resulted in a recovery of about 73 % of intact functional cells in the final adipocyte preparation, used for incubations, and referred to intact WAT (Table 2).

Isolated adipocyte viability

The viability of the cells obtained was high in the final preparation, with a negligible number of cells broken. The incubation of adipocytes resulted in the presence of high concentrations of lactate in the medium. In the incubations, the cells (about 700,000) converted into lactate 37 ± 8 % (triplicates for each of the 4 rats used) of the initial medium glucose after 48 h, and about half this figure at 24 h. The loss of cells was approximately 4 % in the first 24 h and an additional 9 % in the second 24 h period. Otherwise, the glycolytic rate was in the range of 25-35 akat/cell for the whole period, the rates were not different along time when expressed per cell. Consequently, the cells were viable and remained functional for 2 additional days in primary culture.

Analysis of WAT cell type distribution and proportions, cumulative volumes

Table 3 shows the calculations derived from the data of Table 1 to obtain an approximate estimation of the combined proportions of tissue volume filled by the three main types of cells we were able to discriminate: adipocytes, nucleated stromal cells and red blood cells. The total mass of adipocytes was scaled up to the tissue volume from the measured data of mean adipocyte volume and its numbers (estimated from tissue and isolated cells' fat content). Adipocytes constituted almost 0.89 mL/g WAT volume. Total stromal cells and erythrocytes' volumes were, likewise, from their mean cell size and numbers, scaled up to the volume of 1 g of intact WAT. Despite their larger numbers, the combined total volume of all stromal cells accounted for a little more than 2 µL/g WAT.

Using the adipocyte fat content and its volume (both referred to 1 g of tissue weight minus debris), as shown in Table 4, we obtained an approximate estimation of the "live cell mas" of adipocytes in epididymal WAT. This volume includes all the cell organelles, systems and cytoplasm, since the fat vacuole volume corresponds to the fat content, estimated from tissue mass and its direct measurement of fat content. The total cell volume, slightly larger, was calculated from another set of data: cell counting and mean volumes, tracing the cell losses from those of fat. The small difference between both entities is about 3 % of the cell volume, and taken as such is a very small proportion of the whole tissue despite is clearly active metabolism.

Table 5 shows the global distribution of epididymal WAT volume and the space taken up by the three types of cells analyzed. Adipocytes took up 91 % of the tissue volume (excluding the "debris", largely vessels and other structures or undigested tissue), but their number was only 7 % of the total number of cells. Nucleated stromal cells hardly took 0.2 % of the volume but accounted

for 24 % of the cells. Red blood cells were the most abundant, 68 % of numbers, but their space was only 0.05 %, a value that roughly corresponds to 1 µL of whole blood per g of WAT (the rats were exsanguinated, thus this is a residual tissue blood volume). Cells did not occupy all the tissue space, since about 9 % of the tissue volume was extracellular space (interstitial and vascular). Fat alone filled 89 % of the tissue space.

Table 6 summarizes the mean characteristics of the adipocytes extracted from rat epididymal WAT. The estimated non-fat cell volume was in the range of 13 pL, much larger than the 0.29 pL of nucleated stromal cells (Table 3) and the 0.026 pL of red blood cells (Table 1). Adipocytes' "live cell volume" was 45x higher than nucleated stromal or 500x higher than red blood cells. But their complete volume (i.e. including the single fat vacuole was, respectively, 1,600x and 18,000x larger. The combined non-fat adipocyte volume was (Table 5) about one order of magnitude higher than that of nucleated stromal cells. Thus, despite their lower numbers, the mass of "live-cell material" of adipocytes remains the main component of WAT at least using these gross comparison tools.

Discussion

Probably, the most striking conclusion of the present study is the very small proportion of "live cell matter" found in epididymal WAT of normal young adult rats. Fat stores take up an inordinate amount of the tissue space, the interstitial space found is close to that described in previous reports and is in the range of other tissues (Robert & Alemany 1981). However, after excluding the inert fat deposits, the remaining "cell material" accounts for about 3 % of the total tissue mass, which seems very little even in relation to the assumedly limited metabolic activity of the tissue.

It is well known that adipose tissue presents considerable difficulties to work with, the main problem being the dilution of cell proteins, RNA and DNA, as well as its wide variation in almost any parameter, largely attributed to the space occupied by huge fat stores. Evidently, this is not new, but the actual quantification, albeit approximate, of this entity is. The results may seem perhaps extreme, but the combined volume of fat (we often measure the weight, not the volume of fat depots) and extracellular space (i.e. plasma, and interstitial space) markedly limit the possible volume of the sum of blood cells, nucleated stromal cells and adipocyte non-fat cell volume. These considerations support, at least the range of "live cell" volume we have presented here for WAT. It is obvious that the data calculated from the actual experimental results is only an approximation to the real values of this "live-cell" volume of adipose tissue cells. However, the data involved: percentage of fat in the intact tissue, and the combination of mean cell volume and number of adipocytes yield very close figures, with a small difference in cell size over vacuolar fat size. The

different origin of the data, plus the use of different animals to get the means (the individual variability gave too much dispersion), thus we stuck to work with mean values to diminish the noise or clutter of individual data.

In previous works, we have proven the remarkable metabolic activity of the sum of WAT sites (i.e. taken as adipose organ) (Arriarán et al. 2015b; Arriarán et al. 2015c), especially its considerable glycolytic capability (under normoxic conditions) (Arriarán et al. 2015c; Romero et al. 2015), which adds to its known ability to store fatty acids taken from plasma lipoproteins (Garfinkel et al. 1967; Wang et al. 2016) or synthesized from glucose (Guerre-Millo 2003). Its important contribution to amino acid metabolism (Arriarán et al. 2015a), second only to liver (Agnelli et al. 2016; Arriarán et al. 2016), supports the long-proposed active WAT implication in energy and intermediate / substrate metabolism (Cahill 1962). The data presented here only compound the puzzle, since the actual mass of cells doing the work is only a small fraction of the tissue, much lower than usually assumed. The small number of cells (including the stromal nucleated cells) is able to produce a large number of signaling cytokines (Gerner et al. 2013; Wisse 2004), hormones (Killinger et al. 1995; Stimson et al. 2009) and maintain an active capacity to defend (immune system) (Chmelar et al. 2013), and repair or regenerate (i.e. stem cells) (Ogura et al. 2014) tissues. Compared to liver, which cell volume is upwards of 75 % of its volume, the 25-fold lower proportion of WAT "live cell" volume has to show a much higher metabolic activity to be able to carry out the large number of functions and active metabolism that we are discovering in recent times in WAT. The actual quantitation of the mass of adipocyte cytosol and its correlation with metabolic activity is a study worth carrying out, to definitively erase the assumption that WAT is basically an inert dump for excess energy.

Surprisingly, the most abundant cells found in WAT were red blood cells, which accounted for roughly two thirds of the total. The volume of red blood cells was the approximate equivalent to about 1 μL of blood per g WAT, lower than those found previously using ^{65}Fe-labelled red blood cells (Robert & Alemany 1981). Probably, this figure will be higher *in vivo*, since these rats were killed by exsanguination, so that most of the blood was drained. This suggests that *in vivo,* WAT blood content may justify a hefty proportion of its cells.

For operative methodological simplicity, we have analyzed all non-adipocyte cells ("stromal") as a single entity, but we have considered apart, independently, red blood cells, first for their relatively large proportion, and second because of their limited metabolic activity (absence of nuclei). Nevertheless, the combined volume of the nucleated stromal cells was, again, smaller than expected. We are reasonably certain that the methodology used accounted for all free tissue cells in this fraction, since only low-density cells (i.e. adipocytes, and –probably— differentiating preadipocytes) (Grégoire et al. 1990) were separated by the low centrifugation force

used. Our stromal cell data are difficult to compare with the large number of studies available that analyze WAT cell populations under different metabolic conditions, since in practically all cases, the studies are not quantitative, neither referred to initial tissue mass, and are usually centered on preadipocytes (Grégoire et al. 1990), macrophages (Makkonen et al. 2007), vascular (Kajimoto et al. 2010) or other specific cell types (Villaret et al. 2010). In addition, most data on WAT adipocyte counts done in fixed and stained WAT histologic cuts, where, usually, only section areas (of adipocytes) are taken into account, irrespective of the level of the cell at which they have been sliced and then estimated.

In the present study, we expected to find larger numbers of stromal cells, obviously more than blood cells, because this relatively small part of the tissue is responsible for a large number of its metabolic functions and control responses as explained above, and is subjected to considerable variability related to site and inflammation (Cildir et al. 2013; Villaret et al. 2010). In any case, adipocytes remain by large (percentage of WAT volume either counting the fat vacuoles or not) the main component of WAT cell populations, but this primacy was lost when considering the numbers of cells.

One of the critical points this study tried to address was the efficiency of viable cell isolation from freshly dissected WAT. The cell separation method we used is standard, and so widely used that seldom the source is cited, ensuring a fair recovery of the delicate adipocytes with minimal losses. We quantified these losses and found that the recovery was initially close 76 % of the cells initially present in the tissue, but successive manipulations (washings and incubation) resulted in the loss of significant (albeit relatively small) numbers of cells. In any case, we presented a method that allows the establishment of a quantitative relationship between the numbers of functional cells obtained with respect to the initial tissue mass, in the range of 73 %. The data refer to viable cells, able to produce lactate from glucose at an actually high and steady rate, comparable to that of 3T3L1 adipocytes (Sabater et al. 2014).

However, the analysis of recovery was based essentially on the analysis of lipid in all fractions, so that the measurement of volumes (or weights) was critical and introduced a number of factors to be considered for success. First, all cells floating in the buffer after treatment with collagenase and separation of debris (i.e. low density, and preferentially of large volume), were considered adipocytes. Just leaving the cells standing (i.e. centrifugation at 1x*g*) 5 min prevented pressure-caking of adipocytes and their breakage, but allowed a uniform distribution of smaller stromal cells between both phases. This was no problem for their estimation (numbers and volumes), but introduced a possible source of error when using isolated adipocytes for metabolic analysis, since the nucleated stromal cells remained a significant fraction of the crude adipocyte suspension. Three washings resulted in the loss of about 3 % of adipocytes, but theoretically

removed almost all non-attached stromal cells to a negligible proportion of the initial stromal cells content in the adipocyte fraction. The number and volumes of adipocytes found were in the range of those described in the literature (DiGirolamo & Owens 1976; Francendese & Digirolamo 1981). In addition, the cell volumes estimated, combined with the numbers of cells measured accounted for almost all the tissue space available, which is, in itself, an internal check that our calculations and estimations were correct.

Conclusions

The methodology presented here for the estimation of adipocyte recovery allows for a direct quantitative reference to the original intact tissue of studies with isolated cells. This way, the cultured cell data can be used as an approximation to metabolic activity and function related to whole organism.

We have presented proof that the "live cell mass" of adipose tissue is very small. This fact, translates into an extremely high (with respect to the actual "live cytoplasm" size) metabolic activity to justify the overall activity of WAT in glucose-fatty acid relationships, but also in amino acid metabolism. These data justify that comparison of epididymal WAT, often considered the less metabolically active part of the adipose organ, with more metabolically relevant tissues such as liver should take into account these quantitative data, which make WAT an even more significant agent in the control of energy metabolism.

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624

Table 1(on next page)

Results obtained from the collagenase digestion of rat epididymal WAT and the analysis of the tissue and fractions of tissue obtained in the process of separation of viable isolated adipocytes

The data presented as mean ± sem (the data marked with an asterisk * are presented as mean ± sd) are direct experimental data from four different rats

#	parameter	units	values
A1	epididymal WAT weight	g	4.32±0.26
A2	WAT fat content	mg/g	868±8
A3	WAT water content	mg/g	47±3
A4	adipocyte suspension (digested tissue)	g	4.78±0.50
A5	floating fat derived from broken adipocytes	mg	105±55
A6	intact adipocytes suspension (A4 – A5)	g	4.67±0.49
A7	fat in the intact adipocytes suspension	mg/g	537±49
A8	total fat in the intact adipocytes suspension	g	2.51±0.61
A9	water in the intact adipocytes suspension	mg/g	279±23
A10	recovery of intact adipocytes	cells $x10^6$	5.93±2.97 *
A11	adipocyte mean volume	pL	472±285 *
A12	extraction debris mass (dry weight)	mg	357±3
A13	number of total stromal cells freed	cells x 10^6	103±45 *
A14	stromal cells' mean volume	fL	94.6±43.0 *
A15	red blood cells (proportion of A13, total stromal cells)	%	73.7±11.8
A16	red blood cells' mean volume	fL	25.9±1.1 *
dt	intact WAT density	g/mL	0.9402±0.0047
dl	WAT fat density	g/mL	0,9220±0.0112

Table 2(on next page)

Analysis of the effectivity of the adipocyte isolation procedure used based on the analysis of lipid distribution, from intact tissue to the final preparation of adipocytes

The data are mean values calculated from the experimental data in Table 1. The column "calculations" explains the data used in each case.

Volumes were calculated with dt or dl (Table 1) when applied to tissue (V = W / dt) or lipid (V = W / dl), where W is weight (in g) and V volume (in mL). In the calculations marked (W and V), the values were calculated directly from weights and volumes, i.e. not applying the density factors.

#	parameter	weight mg/g intact WAT	volume µL/g intact WAT	calculations
B1	intact epididymal WAT	1000	1064	
B2	extraction debris (dry weight)	83±1	88	(A12 x B1) / A1
B3	WAT fat content	868±8	942	A2
B4	WAT mass minus debris	917	976	B1 – B2 (W and V)
B5	WAT fat content corrected by debris	796	864	(B3 x B4) / B1 (W and V)
B6	lipid, from broken adipocytes, in the fat layer	24	26	(A5 x B1) / A1
B7	total WAT fat in the extracted adipocytes	772	837	B5 – B6 (W and V)
B8	total fat in the intact adipocytes recovered	581	630	(A8 x B1) / A1
B9	total fat in the adipocytes recovered (intact or broken)	605	657	B6 + B8 (W and V)
B10	fat loss during adipocyte isolation	191	207	B5 – B9 (W and V)
B11	percentage of adipocyte fat recovery	*76.0*	--	(B9 / B5) x 100
B12	percentage of adipocytes (fat) lost in the fat layer	*3.1*	--	(B6 / B5) x 100
B13	percentage of intact adipocytes (expressed as fat) in the final	*73.0*	--	(B8 / B5) x 100

Table 3(on next page)

Calculation of the volumes of cells from rat epididymal WAT

Data calculated using the experimental results presented in Tables 1 and 2.

#	parameter	units	values	calculations
C1	adipocytes in WAT	cells x 10^6/g WAT	1.88	(B5 / B1) x (A10 / A8)
C2	combined volume of WAT adipocytes	µL/g WAT	888	(A11 x C1) / 10^6
C3	stromal cells in WAT	cells x 10^6/g WAT	23.9	A13 / A1
C4	red blood cells in WAT	cells x 10^6/g WAT	17.6	(C3 x A15) / 100
C5	nucleated stromal cells in WAT	cells x 10^6/g WAT	6.3	C3 – C4
C6	total volume of stromal cells in WAT	µL/g WAT	2.3	(C3 x A14) / 10^9
C7	total volume of red blood cells in WAT	µL/g WAT	0.46	(C4 x A16) / 10^9
C8	total volume of nucleated stromal cells in WAT	µL/g WAT	1.80	C6 – C7
C9	mean nucleated stromal cell volume	fL	287	(C8 / C5) x 10^9

Table 4(on next page)

Calculation of the non-fat cell volume of adipocytes in rat epididymal WAT

Data calculated using the experimental data presented in Tables 1-3.

#	parameter	volume (µL / g WAT)	% of total cells volume	calculations
D1	total volume of adipocytes in 1 g of WAT	888	100	C2
D2	total fat volume in 1 g of WAT	864	97.3	B5
D3	non-fat adipocyte cell volume in 1 g of WAT	24	2.7	D1 – D2

Table 5(on next page)

Distribution of cell types by volume and number in rat epididymal fat

Data calculated using the results presented in Tables 1-4.

#	parameter	volume µL/g WAT	% of WAT volume	cells (10^6/g WAT)	% of WAT cells	calculations
E1	initial WAT weight (minus debris)	976	100.0			B4
E2	adipocytes	888	91.0	1.88	7.3	D1
E3	red blood cells	0.46	0.05	17.6	68.3	C7
E4	nucleated stromal cells	1.80	0.19	6.28	24.4	C8
E5	total cells	890	91.2	25.8	100.0	E2 + E3 + E4
E6	extracellular space	86	8.8			E1 – E5
E7	fat	864	88.5			D2
E8	total stromal cell volume	2.3	0.23			C6
E9	total nucleated cell volume	890	91.2			E2 + E4
E10	adipocyte non-fat cell volume	24.1	2.5			D3

Table 6(on next page)

Characteristics of the adipocytes isolated from rat epididymal adipose tissue

Data calculated using the results shown in Tables 1-3.

#	parameter	units	values	calculations
F1	lipid content	mg/g	796	B5
F2	cell lipid weight	ng/cell	423	B5 / (C1 x 10^6)
F3	cell lipid volume	pL/cell	459	(B5 / C1) x 10^6 /dl
F4	cell mean volume	pL/cell	472	A11
F5	non-fat cell volume	pL/cell	13	F4 – F3

Figure 1

Representative microphotography of an adipocyte preparation observed at the microscope using a Neubauer chamber

The squares in the grid have a width of 250 µm, and correspond to a volume of 6.25 nL

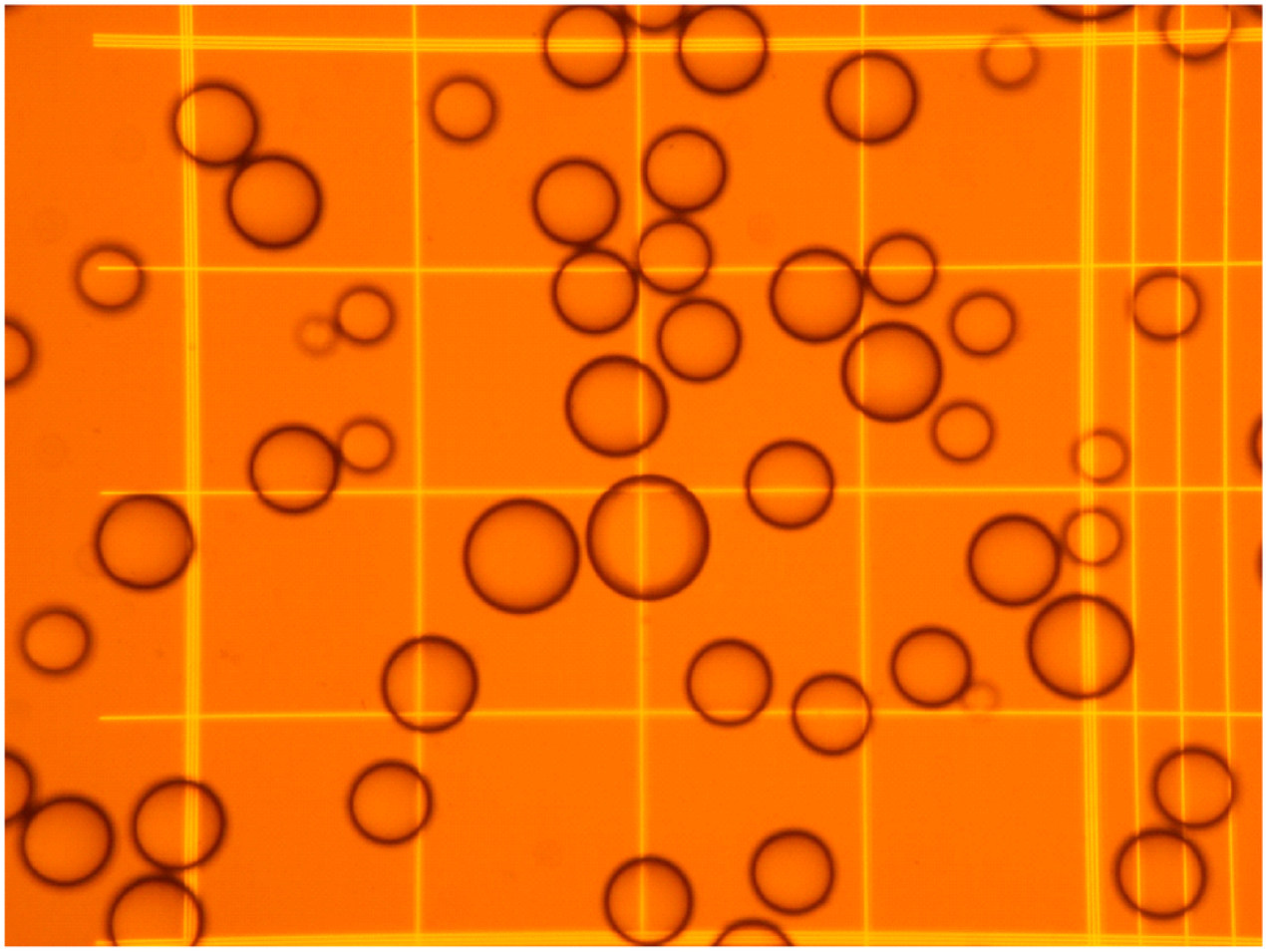
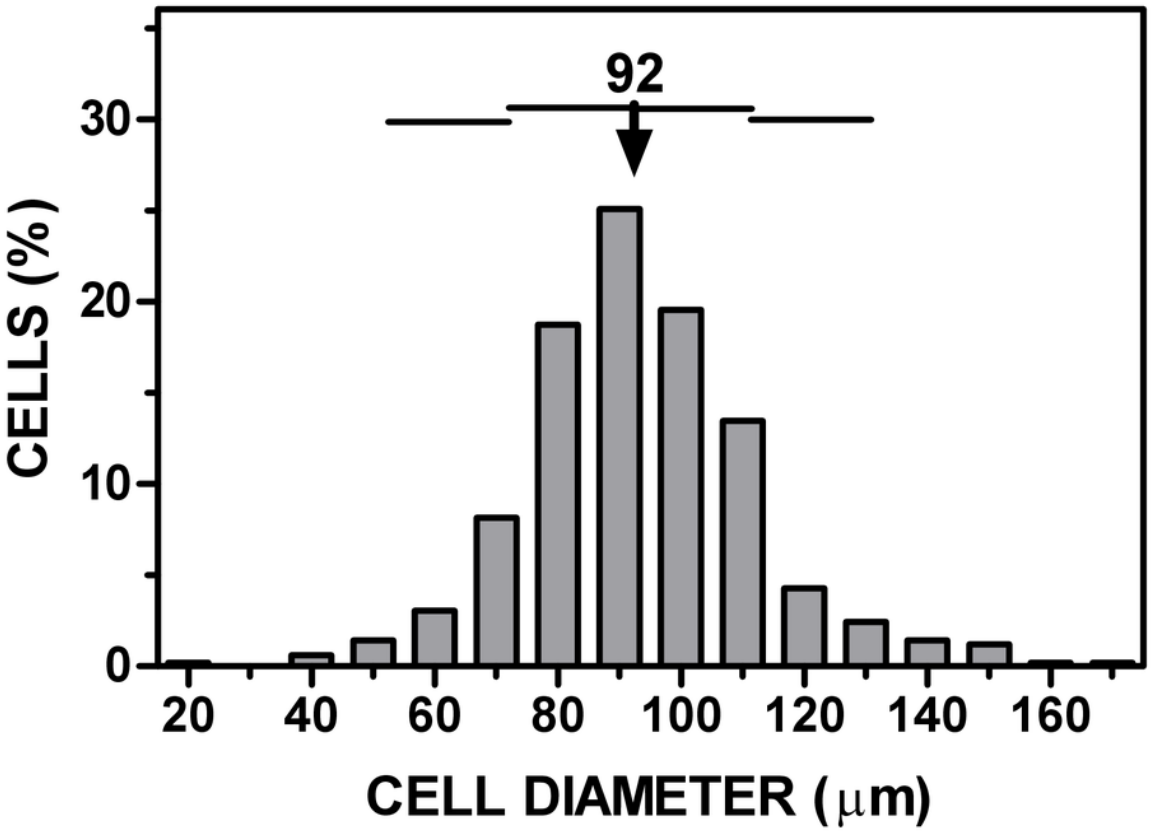


Figure 2

Representative graph of cell size (diameter, volume) *vs*. cell numbers representation obtained applying the cell extraction procedure described in the text to a sample of epipidymal adipose tissue

The data have been grouped to facilitate the presentation. The arrow (and the number above) represent the mean cell diameter and volume. The horizontal lines represent each one the extent of one sd.


92
CELLS (%)
0
10
20
30
20
40
60
80
100
120
140
160
CELL DIAMETER (μm)

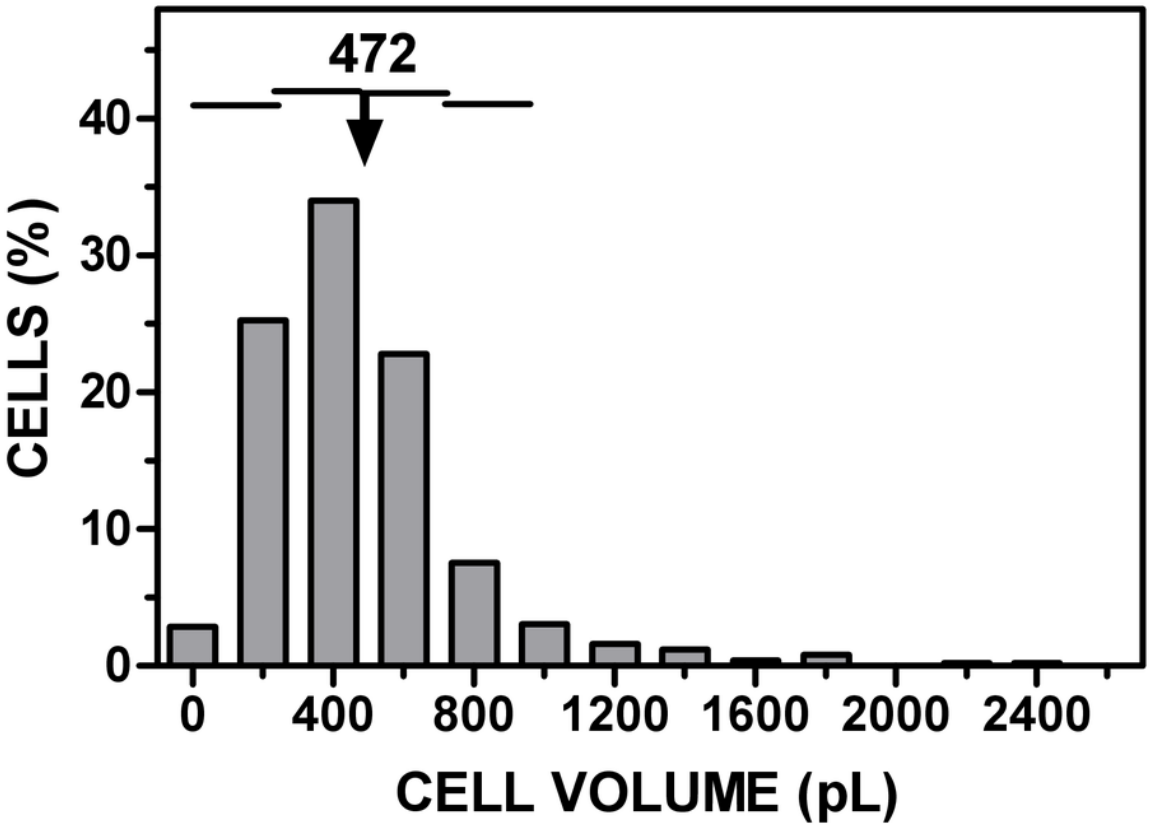
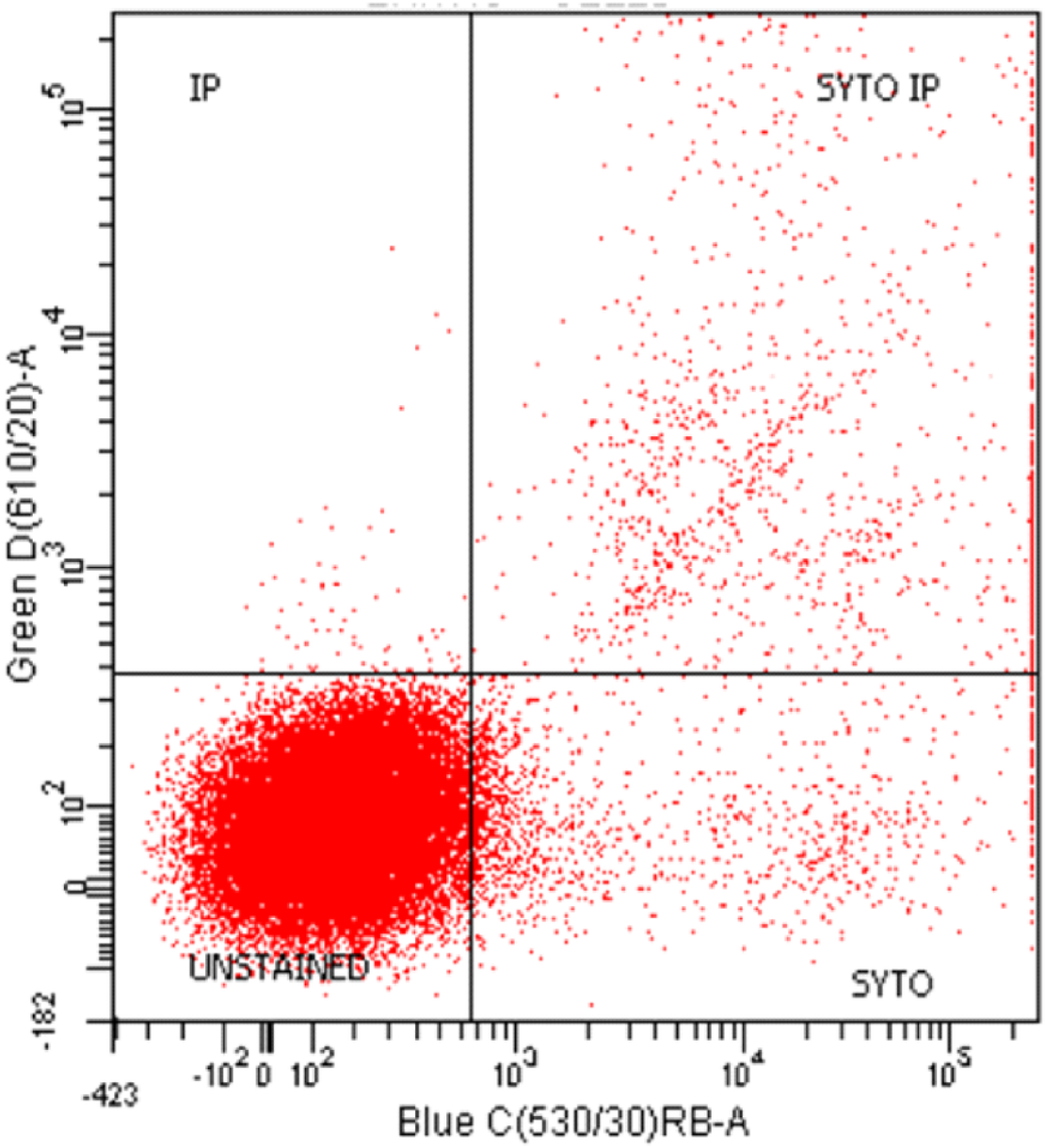

472
CELLS (%)
0
10
20
30
40
0
400
800
1200
1600
2000
2400
CELL VOLUME (pL)

Figure 3

Representative graph of flow cell analysis of stromal fraction of epididymal rat WAT to discriminate nucleated from non-nucleated cells.

Both propidium Iodide (IP) and Syto-13 (SYTO) bind DNA-positive and double positive particles (i.e. cells). The dots in the upper and right areas correspond to nucleated stromal cells; dots in the lower-left square show the unstained cells, largely corresponding to the high proportion of erythrocytes.


IP
SYTO IP
UNSTAINED
SYTO
Green D(610/20)-A
Blue C(530/30)RB-A
-182
-423
10^5
10^4
10^3
10^2
0
-10^2
10^2
10^3
10^4
10^5