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Culture of Isolated Human Adipocytes and Isolated Adipose Tissue

Kirstin A. Carswell, Mi-Jeong Lee, and Susan K. Fried

Abstract

Adipose tissue (AT) is no longer considered merely as insulation or padding for human organs. It is an endocrine organ in its own right, which includes composite cells with the ability to differentiate into multiple cell lines.

In fact, there is increasing evidence to support the theory that the causation of obesity and its associated metabolic disorders originate at the cellular or tissue level. Adipocyte dysfunction and chronic inflammatory states are able to modulate triglyceride storage and mobilization directly through cytokine and adipokine release.

Significant variability exists between adipocyte isolation and culture techniques which subsequently can impact experimental results. We aim to explain the importance of controlling these variables, to assist tailoring methodological choice towards the investigational outcomes, and modifications of the techniques used accordingly.

The techniques described in this chapter yield cell and adipose tissue which can be utilised in many different ways, including adipose tissue stem cells for differentiation, DNA analysis, RT-PCR, immunohistochemistry, lipolysis, glucose uptake, and LPL activity.

Keywords

Adipocytes; Adipose tissue; Cell culture techniques

1. Introduction

1.1. The Role of Adipose Tissue

The functions of adipose tissue (AT) have not been fully elucidated. Adipocytes store energy as triglycerides, the main energy source for the body, and release it in the form of non-esterified fatty acids, when required (1). AT also releases multiple cytokines (2), some with local paracrine effects, e.g. TNF- α and IL-6 which have pro-inflammatory effects (3–5). In addition, adipocytes can respond to and secrete hormones, e.g. leptin and adiponectin as well as acylation-stimulating protein and others, which are of paramount importance in the regulation of energy intake and expenditure as well as systemic inflammation and metabolism (6). This suggests that adipose tissue is the largest, and potentially one of the most important, endocrine organ in the body (7).

Adipose-resident stem cells acquired from collagenase digestion of adipose tissue are multipotent with the ability to differentiate into many different cell lines, including adipocytes, bone, cartilage, endothelium, neural (8, 9), and liver cells (10–12). This, combined with their ease of harvesting, suggests their potential as the most important adult stem cell reservoir (13).

1.2. Adipocytes Versus Adipose Tissue

Whether to use adipose tissue or adipocytes in your experiments is a decision which should not be taken lightly. Other cells, especially macrophages can adhere to isolated fat cells (14). Thus, primary adipocytes (fresh or cultured) are useful for acute metabolic studies; however, caution is advised if using isolated cells for other goals.

Organ culture by its nature contains other cells, including preadipocytes, endothelial cells, and fibroblasts and various immune cells (15). Despite this, many believe organ culture to offer substantial benefits over adipocyte culture. It is the preferred method when assessing the long-term regulation of gene expression and adipocyte function within the AT (16–18) as adipocyte-specific gene expressions in the human AT are maintained (19). However, loss of adipocyte-specific gene expression, e.g. GLUT4, and loss of insulin sensitivity have been reported in isolated rodent adipocyte culture (20). Adipocytes isolated from these fragments remain responsive to acute hormonal effects after culture (21, 22). Therefore, studies of the mechanisms involved in the long-term regulation of genes important for studies of insulin resistance, type 2 diabetes mellitus, and obesity, can be performed (17, 23). Encouragingly, this methodology appears to correlate well with in vivo effects (15).

1.3. Regional Variations in Adipocyte Biology

Variations in regional distribution of body fat has been linked with metabolic and cardiovascular disease (24). Increased visceral adiposity in particular has been correlated with hyperlipidaemia, insulin resistance, and T2DM (25). Other studies also suggest heterogeneity among subcutaneous, abdominal, and gluteal depots (26–28).

Significant variations exist not only in AT distribution, but the molecular characteristics of the adipocytes at these depots. Morphological and functional differences have been reported between visceral and peripheral AT (29), e.g. hormone receptor expression, adipokine secretory profile, and expression pattern (30). In addition, depots vary with respect to adipocyte cell size or different cell types present (28, 31, 32). This may be due to different laboratory preparatory techniques or the comparatively small sample sizes used.

Interestingly, visceral adipose tissue have been shown to express more genes encoding pro-inflammatory cytokines than subcutaneous adipose tissue (32–34) which may or may not translate to the protein level. Depot-related differences in adipose-produced molecules have also been reported, including leptin, adiponectin, IL-6, and angiotensinogen (35, 36), with an increase in IL-6 secretion in visceral cells (37) but no difference in TNF- α secretion (33). Variation also has been reported in receptor expression between subcutaneous adipocytes from different sites. Subcutaneous abdominal adipocytes have larger numbers of stimulatory beta adrenergic receptors on the cell surface and higher stimulatory lipolytic activity when

compared to gluteal (38) presumed mainly due to higher inhibitory alpha 2 adrenergic receptors in gluteal versus abdominal adipocytes (26, 39).

In response to this, much research is targeted towards identifying the differing roles of adipocytes within their respective depots and potential differences in gender, age, ethnicity, and metabolic disorders (37, 40–42).

2. Materials

2.1. Adipose Tissue Collection and Handling

1. Sterile scalpel and scissors.
2. Sterile 50-cc falcon tubes (see Note 1).
3. Transport medium: 0.9% Saline, Phosphate Buffered Saline (PBS) solution or Medium 199 (M199) (Gibco) with glutamine at room temperature (RT).

If being used for cell or organ culture, add penicillin (100 U/l), streptomycin (100 µg/ml), and gentamicin (50 µg/ml) to the transport medium.

2.2. Adipocyte Isolation

1. Sterile 50-cc polypropylene tubes.
2. Sterile scissors, forceps, and perforated spoons.
3. 95% O₂:5% CO₂ gas.
4. Parafilm®.
5. 37°C water bath.
6. 240–300 micron nylon mesh (Small parts or Cole-Palmer).
7. Polypropylene funnel (see Note 1).
8. Polypropylene syringes (10 and 20 ml).
9. Elastic bands.
10. Polypropylene tubing (VWR).
11. Polypropylene Erlenmeyer flask.
12. Krebs-Ringer Bicarbonate buffer (KRB) or M199.
13. Fatty acid-free and endotoxin-free bovine serum albumin (BSA) (see Note 2).
14. D-glucose (Sigma).
15. 200 nM Adenosine or N⁶-(1-methyl-2-phenylethyl) adenosine (PIA) (Sigma).

¹Fat cell samples should not be kept in glass vessels as this can result in lysis of mature adipocytes.

²BSA helps maintain the integrity of adipocytes and prevent breakage.

16. Collagenase (Type 1) solution: 1 mg/ml Type 1 Collagenase (Worthington) or Liberase Blendzymes (Roche) in KRB (or M199) with 4% BSA (and 200 nM adenosine or PIA) (see Notes 3 and 4).

2.3. Culture Technique

2.3.1. Adipose Tissue Organ Culture

1. AT samples obtained from human subjects (see Note 5).
2. Laminar flow hood.
3. Tissue culture incubator, 5% CO₂ atmosphere.
4. Sterile 50-cc tubes.
5. Sterile scissors, forceps, and perforated spoons.
6. 100-mm-diameter sterile Petri dishes, 60-mm and 35-mm culture dishes.
7. PBS or 0.9% normal saline.
8. Culture medium: M199 (Gibco), 50 µg/ml gentamicin added (see Note 6).
9. Insulin (recombinant human, e.g. Humulin 100 U/ml).
10. Glucocorticoids (dehydrocortisone or dexamethasone (DEX)).
11. 240–300-µm sterile polypropylene or nylon mesh affixed on top of funnel and autoclaved.
12. Sterile pipettes (10 and 25 ml).

2.3.2. Isolated Mature Adipocyte Culture

1. Laminar flow hood.
2. Tissue culture incubator, 5% CO₂ atmosphere.
3. 240–300-µm sterile polypropylene or nylon mesh affixed on top of funnel and autoclaved.
4. Sterile 50-cc tubes (polypropylene or polystyrene).

³Variability exists between lots in the enzyme activity and performance of the type 1 collagenase used. As such, pre-testing of specific lots should be performed prior to purchasing in bulk for all experiments used in the series.

⁴The addition of adenosine or PIA prevents cell breakage and high lipolytic rates.

⁵Full research ethics permission must be acquired prior to removal of human tissue specimens. Participants must have given fully informed consent, and appropriate storage facilities must be available.

⁶The use of albumin-free conditions or addition of albumin or serum, e.g. 0.5–5% FBS, for culture media is debatable.

Supplementation, or not, of serum may impact upon experimental outcome as it contains many growth factors (with inter-lot variability).

Serum-free media is recommended for AT organ culture since mature adipocytes do not require serum for their maintenance, and BSA or FBS increases cytokine expression while decreasing adipocyte gene expression, such as LPL, during the culture (our unpublished observation). However, in some culture conditions (e.g. addition of free fatty acids), it is necessary to supplement additional BSA or FBS due to deliver to free fatty acids.

It should also be noted that proliferation of stromal fibroblasts (which can convert to adipocytes) within adipose tissue has been reported in >5% serum-containing media (43).

5. Culture medium: M199 or Dulbecco's modified Eagle medium (DMEM) with Ham's F12 (Gibco) with gentamicin solution (50 µg/ml) ± foetal bovine serum (FBS) or BSA (see Note 6).
6. Insulin (recombinant human, e.g. Humulin 100 U/ml).
7. Glucocorticoids (dehydrocortisone or DEX).
8. Sterile pipettes (10 and 25 ml).
9. 100-mm sterile Petri dishes.
10. Sterile scissors and forceps.

3. Methods

3.1. Adipose Tissue Collection: Adipose Tissues Are Generally Obtained by Surgical Resection or Subcutaneous Adipose Tissue Aspiration

1. Under general or local anaesthesia, the skin is cleaned with aseptic preparation.
2. Skin incisions are made using a scalpel and the required volume of adipose tissue excised using either scalpel or scissors (see Note 7).
3. Careful consideration must be made with respect to specimen extraction in advance (see Note 8).
4. If the adipose tissue is to be needle aspirated, this can be performed directly using a wide-bore (2.5 mm, 1–3-hole cannula) needle into a sterile 50-cc syringe.
5. AT biopsies (obtained through either technique) are placed into capped, sterile 50-cc tubes containing M199 as transport medium (RT).
6. Specimens are transferred to the laboratory as soon as possible, within 30 min.
7. Any surplus tissue can be snap frozen in the operating theatre or clinic and stored at –80°C for future use.

3.2. Adipose Tissue Processing: Sterile Conditions Are Required

1. Perform the following procedures under a laminar flow hood. Mince the tissue into small pieces, approximately 5–10 mg per pieces (1–2 mm³), using sterile sharp scissors (see Note 9). Adipose tissue obtained from needle aspiration is already fragmented and does not require further mincing.
2. Pour minced tissue through a nylon mesh, affixed to a funnel and placed on top of a 500-ml bottle to capture the waste.

⁷Avoid using diathermy or harmonic scalpel as this can result in damage to the cells being sampled; however, samples aspirated with mini liposuction have been used previously.

⁸Should it be necessary to extract the specimen through another adipose tissue depot, e.g. omental AT must pass through subcutaneous AT layer, an Endobag™ or equivalent should be used to avoid contamination at this point.

⁹Mincing should be performed using a sharp implement, e.g. scalpel or scissors, to avoid crushing of the cells. This can be performed using a two-handed scissor technique in plastic, conical, 50-cc tubes.

3. Pour room-temperature saline or PBS over the tissue on the funnel to remove broken cell debris and lipid. Several tubes of minced tissue can be combined on the funnel and washed together. Remove any visible blood clots and connective tissues using sterile forceps.
4. Transfer the tissue into a pre-weighed sterile Petri dish using forceps or perforated spoons and weigh the Petri dish with tissue to get tissue weight. Since the tissue is not quite dry, the actual weight of the tissue is overestimated by about 10–20%.
5. The AT can be used for adipocyte isolation or adipose tissue organ culture.

3.3. Adipocyte Isolation

1. The AT from Subheading 3.2 is placed into 50-cc tubes containing 1 mg/ml Collagenase (Type 1) solution. Generally, 2–3 ml of collagenase solution per 1 g adipose tissue is used. There may be variations in collagenase and BSA obtained from different companies and different batches (see Notes 2 and 3).
2. Place the tubes into the cell culture incubators loosely capped and equilibrate for 15 min.
3. Tightly cap the tubes and incubate in a 37°C water bath for 30–60 min with shaking at 100 rpm until the mixture has a “soupy” consistency (see Note 10). Gently swirl the tubes every 15 min or so and check the degree of digestion.
4. Transfer the mixture into the laminar flow hood and proceed to next steps.
5. Gently pour the mixture through a nylon mesh filter affixed on top of funnel and placed on top of a 50-cc tube (see Note 11). Wash mesh with culture media. Separate the adipocyte fraction from stromal vascular fractions. This can be achieved by removing the lower fraction below adipocytes after centrifuging at low speed ($500 \times g$ for 1 min) or simply floating adipocytes to the top of tubes. Wash adipocytes with culture media three times (see Note 12).
6. Resuspend the adipocytes in M199 or DMEM:F12 without or with supplementation with BSA or 5% FBS (see Note 6). The volume of fat cell yield is recorded and then diluted in 1:10 ratio.
7. Adipocyte (see Fig. 1) cell size and number should be calculated using established protocols (see Note 13).

¹⁰The specimen should not be left in the Collagenase solution for longer than 60 min as this reduces adipocytes' viability.

¹¹This can be performed using end-cut syringes, nylon meshes, and rubber bands. Gently pour the digested AT inside the “end of syringe”, place a mesh over the tip, and secure this with an elastic band. While wearing protective clothing, including goggles, the cell solution may then be passed through the mesh into a polypropylene flask using a funnel. Alternatively, the nylon mesh can be affixed on top of funnel and the cell solution poured through this into 50-cc tubes.

¹²The adipocytes are washed by pouring media indirectly into the tubes, mixing with polypropylene tubing, and slowly aspirating the supernatant from below the fat cell layer using blunt-end needle connected to a syringe, thereby minimising disruption of the cells. Precursors of adipocytes can be obtained by centrifuging the first aspirate at $500 \times g$ for 5 min. Cell pellets can be plated and expanded in α -MEM supplemented with 10% FBS (44, 45). These cells can be differentiated into adipocytes in vitro.

¹³Cell sizing can be performed using a Coulter counter or light microscopy. If light microscopy is used, digital images at $\times 4$ magnification can be transferred into Image J software (<http://rsb.info.nih.gov/ij/>). This software can be used to calculate the average diameter of >200 adipocytes (46). Total triglyceride determination is performed using Dole's lipid extraction and adipocyte cell number is extrapolated as per Di Giralomo (47).

3.4. Culture Technique

3.4.1. Adipose Tissue Culture—This procedure is undertaken in a laminar flow hood.

1. Calculate the potential number of dishes based on the total tissue weight from the procedure 3.2 (~0.5 g/100 mm or smaller if required, keeping the proportion of tissue to media (150–250 mg/60-mm or 50–100 mg/35-mm dishes) at approximately 30 mg/ml¹⁵) for use in experimentation (see Note 14).
2. Calculate and prepare culture media (see Note 6) with appropriate hormones, chemicals, and other additions added.
3. Distribute the tissue into culture dishes and add pre-warm culture media [15 (100-mm dish), 5–7 ml (60-mm dish), or 2–3 ml (35-mm dish) with appropriate hormones or other additions]. In our experience, the combination of insulin and glucocorticoids maintains adipocyte gene expression similar to freshly obtained tissues.
4. Incubate in cell culture incubator at 37°C under 5%CO₂/95% air atmosphere.
5. Replenish culture media every 2–3 days by aspirating using serological pipettes (see Note 15). This removes adipose tissue metabolites and secretory products (free fatty acids, glycerol and cytokines, etc.) which accumulate during the culture. This also ensures maintenance of hormones that are degraded during long-term culture.
6. Generally, you can maintain AT in organ culture for 7–9 days in vitro (see Notes 16 and 17). It is worth noting that during the early days of cultures (1–2 days), the inflammatory cytokine levels are extremely high while LPL activity is almost null compared to the levels in freshly obtained AT and after days 6–7 of culture their levels are restored.
7. After culture, weigh the amount of tissue in each plate and freeze or use for other experiments.

3.4.2. Adipocyte Culture—This procedure is undertaken in a laminar flow hood.

1. Adipocytes are isolated and resuspended in the culture media using the protocol described in 3.1 with antibiotics added (see Note 6).
2. Resuspend adipocytes ~10% (i.e. 1-ml packed cells into 10-ml culture media) (see Note 6). FBS or BSA can be used for isolated fat cell culture to capture free fatty acids released during the culture (see Note 4).

¹⁴For organ culture, it is crucial that the fragments are ~2 mm³ as necrosis of internal portions of AT has been noted <24 h when pieces exceeded 4–5-mm diameter (48). It is also important to have uniform-sized tissue pieces to minimise your experimental errors. More than 0.5-g tissue/dish (100 mm) decreases maximum LPL activity that can be induced by insulin and DEX and may affect other aspects of adipocyte function (15).

¹⁵When exchanging media, care should be taken to avoid touching the dish. Slowly aspirate the media below the floating adipose tissue fragments using serological pipettes.

¹⁶Time courses may vary depending upon the adipose tissue function being assessed.

¹⁷Basic experimental outcomes using this technique include quantification of the glycerol and adipokine/cytokine content of the media and tissue analysis for LPL activity, mRNA, and protein.

3. Distribute the adipocyte suspension into culture plates or 50-cc tubes (should be loosely capped during the culture). Mature adipocytes float easily and gentle swirling is required during the dividing process to get equal number of cells per plate or tube.
4. Place the tubes or dishes in a cell culture incubator.
5. Exchange media after first 24 h and then alternate days (see Note 18). Since fat cells float on top of plates or tubes, gently aspirate culture media using 1- or 5-ml pipettes and add new culture media gently avoiding direct shooting into adipocytes or contact with them (see Note 19).

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¹⁸This media exchange technique differs from other media exchange techniques as adipocytes have a tendency to float. As such, the pipette is placed through the media to the bottom of the dish, avoiding touching the base. The media is slowly aspirated and ~0.5–1-ml residual can be left if necessary to facilitate this. Fresh media should be added in a similarly gentle fashion to minimise disruption to the cells.

¹⁹To optimise the insulin response of cells, reduce the glucose concentration in the medium for a few days before experiments.

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Fig. 1.
A representative example of human adipocytes isolated from adipose tissue.