Determination of adipocyte cell size by H & E stained adipose tissue and collagenase digested isolated adipocytes

Devaligoda Gamage Kalpani Yashodara Perera*, Hemantha Senanayake2, Tharanga Thoradeniya1
1Department of Biochemistry and Molecular Biology, Faculty of Medicine, University of Colombo, Sri Lanka.
2Department of Obstetrics & Gynaecology, Faculty of Medicine, University of Colombo, Sri Lanka.
*For Correspondence : kalpaniperera2013@gmail.com

Abstract
The aim is to compare the two methods H & E stained adipocytes and collagenase digested isolated adipocytes, and determine if collagenase digested isolated adipocytes analysis is an accurate method for cell size estimation. The adipose tissue samples (superficial subcutaneous adipose tissue; sSAT, deep subcutaneous adipose tissue; dSAT and visceral adipose tissue; VAT) were collected from three pregnant women undergoing caesarian section. The adipocyte size was determined in collagenase digested isolated adipocytes and H & E Staines adipocytes by ImageJ software and the two methods were compared. There were no significant difference in the two methods and was strongly correlated (r=0.99, P<0.01). The bias was observed between the collagenase digested isolated adipocytes and H & E stained adipose tissue since 97.48% of the total adipocytes was within the limit of agreement. In conclusion the collagenase digested isolated adipocytes can be used as an accurate and less cumbersome method for the cell size determination.

Key words : Adipose tissue, H & E stained, isolated adipocytes, method comparison

Abbreviations
sSAT: superficial subcutaneous adipose tissue, dSAT: deep subcutaneous adipose tissue, VAT: visceral adipose tissue, H & E: Hemotaxylin and Eosin.

Introduction
Adipose tissue is known to be a highly metabolically active tissue, which is important for regulation of energy intake and expenditure, lipid metabolism and creates systemic inflammation (5, 7, 13). Adipocyte size is known as an indicator of the adipose tissue expansion during weight gain, obesity and also during the process of pregnancy (17). The AT expansion takes place in two phases. The first phase is hypertrophy, wherein the adipocytes increase in size. Secondly, hyperplasia takes place where precursor cells known as preadipocytes are formed (2, 12, 20). Determination of adipocyte size is essential for metabolic studies, to determine endocrine functions and to detect the changes in adipose tissue morphology (4, 8, 19).

The adipocyte size measurement have been commonly done on stained tissue (4, 12) as well as collagenase digested isolated adipocytes (3,21)although drawbacks are observed in both methods. The collagenase digested isolated adipocytes must be determined immediately after Isolation, if the cell suspension is stored it causes changed in the cell morphology (3) such as distortion in larger isolated adipocytes (4). Furthermore, since the isolated adipocytes are
unfixed and floating the same adipocyte may be captured more than once (4). The histological slide preparation has the ability to minimize the morphological changes and the cells overlapping when compared with collagenase digested isolated adipocytes (11). The main drawback of histological slide preparation is the need of well-trained personals. Moreover, histological sample preparation is more time consuming (19) compared to collagenase digested cell isolation which is a less expensive and a faster method (21). Thick histological preparations (19) and prolonged storage in 10% formalin may cause the shrinkage of the adipocytes (3).

Our objective was to compare the two methods H & E stained adipocytes and collagenase digested isolated adipocytes, and determine if collagenase digested isolated adipocytes analysis is an accurate method for cell size estimation.

Materials and methods

Adipose tissue collection: The adipose tissue samples were collected from three pregnant women undergoing caesarian section at De Soysa maternity hospital. The study procedure was approved by the Ethics Review Committee of the Faculty of Medicine, Colombo. Permission was granted by the director of the De Soysa maternity hospital.

The adipose tissue samples were collected under surgical conditions with sterile equipment. The samples were collected from pregnant women while performing lower segment caesarian section and after the delivery of the child. The samples of the adipose tissue (superficial subcutaneous adipose tissue; sSAT, deep subcutaneous adipose tissue; dSAT and visceral adipose tissue; VAT) were collected from the lower quadrant 10-12cm from the umbilicus in the hypogastric region. The VAT was collected below the rectus fascia. The sSAT was collected just below the skin and the dSAT was collected from just above the rectus fascia. All the samples were collected in phosphate buffered saline (PBS) and transferred into the laboratory within 30 minutes.

Determination of adipocyte size in collagenase digested isolated adipocytes: The isolation of the adipocytes was done by the method described by Rodbell (1964). Before the isolation blood vessels were removed from the adipose tissue using a forceps and a scalpel. For the adipocyte isolation 100mg of adipose tissue was suspended in 1ml of Krebs-Ringer bicarbonate buffer (137 NaCl, 5 KCl, 4.2 NaHCO₃, 1.3 CaCl₂, 0.5 MgCl₂, 0.5 KH₂PO₄, 0.5 MgSO₄, 20 HEPES (pH 7.4) and 1% BSA) and to this 5 mM glucose and 1 mg/ml collagenase (SIGMA catalogue no.C0130) was added. The digestion was carried out at 37 °C (water bath) with constant shaking for 45 minutes. Cells were filtered through a cloth mesh and washed three times with Krebs-Ringer bicarbonate buffer. The suspension is centrifuged for 1 minute at 400 x g. The fat cells floated in the surface, while the stromal-vascular cells (capillary, endothelial, mast, macrophage, and epithelial cells) were sedimented. The pellet was removed and the fat cells were resuspended in 1ml of Krebs-Ringer bicarbonate buffer containing glucose and it is centrifuged for 1 minute at 400 x g. This procedure was repeated three times. By the end of this centrifugation process the stromal-vascular cells were absent and the fat cells were found floating in the surface of the tubes. About 30μl of adipocytes cell suspension was placed on a glass slide and the area was covered with a cover slip. The cell image was captured using the 10x objective of the Olimpus 1x70 fluorescent inverted microscope. Successive images (50 images) were taken while assuring that the images were not captured previously.

Determination of cell viability in the collagenase digested isolated adipocytes: The cell viability was measured in adipocyte suspension by trypan blue staining. The cell viability was assayed just after the cell isolation and after 3 hours. A drop of trypan blue was mixed with 100μl of cell suspension and left for 1-2 minutes for absorption. The suspension was loaded to the hemocytometer and the total cell number and the stained cell number was counted.
The percent of cell viability was detected by the following equation:

\[
\text{Percent viability} = \frac{\text{Total cells counted} - \text{stained cells}}{\text{Total cells counted}}
\]

**Determination of adipocyte size in stained adipose tissue**: The adipose tissue was fixed in 10% formalin and stained using the H & E stain (18). Firstly the samples are paraffin embedded using cassettes. The sample is cut into 5μm thin tissue and then the slides are prepared. For the staining the following steps are followed: deparaffinization, dehydration, staining with Harri’s Hematoxylin solution followed by washing and dehydration and counter staining by eosin Y solution. After the slides were prepared 10 successive images were captured using the 10x objective of the Olimpus 1x70 fluorescent inverted microscope.

**Determination of adipocyte size using ImageJ software**: After the capturing of the images the ImageJ software (http://imagej.nih.gov/ij/) was used for the quantification of the adipocyte cell size. ImageJ is a free software which can be used for manual counting of cell number and size.

Before the ImageJ was used to determine the cell size the software was calibrated using an image captured from the same microscope with a known scale. After the calibration the image of the adipocytes were opened in the software and converted to a grey scale image. The background noise of the image was removed to improve the clarity of the image. A threshold image was prepared to identify the membrane material and the empty space in black and white. The unwanted particles were removed and the specific adipocytes were chosen to determine the adipocyte size. Finally, the adipocyte size was measured using the cell surface area (μm²) using the criterion’s mentioned below. Cells having an area below 150μm² were excluded since they may include stromal vascular cells and cells having an area more than 50,000 μm² were also excluded because they might be distorted, the shape factor of the adipocytes was placed between 0.35-1 (In this 1 represent a perfect circle while 0 represents a straight line), adipocytes touching the border of the image were excluded and larger adipocytes which were shrunken were excluded. For the analysis 150 adipocytes was used (14, 15, 23).

**Statistical analysis**: To compare the two methods H & E stained adipose tissue and collagenase digested isolated adipocytes several statistical tests were performed. Student’s paired t-test was performed to observe the difference in the two methods. The spearman’s correlation and scatter plots were used to assess the association and the linear relationship of the methods. The Bland-Altman plots (1) were used to assess the existence of bias between the two methods. For this the difference between the methods (Bias=isolated adipocyte size-stained adipocyte size) were plotted on the y axis against the average of the method on the x axis. All the statistical analysis were performed using SPSS (Statistical Package for Social Sciences) version 18.0 statistical software (SPSS Inc., Chicago, IL, USA) and Microsoft Excel 2010 was utilized.

**Comparison of adipocyte size in pregnant women**: Apart from the method comparison the cell surface area (μm²) of the adipocytes was compared in two groups of pregnant women (3 normal weight pregnant women and 3 overweight pregnant women) utilizing the isolated adipocytes methodology. The cell size of the two groups was compared utilizing student’s t-test and Spearman’s coefficient.

**Results**

To compare the size of collagenase digested isolated adipocytes and H & E stained adipose tissue a total of 9 adipose tissue depots were used with a total of 1350 adipocytes. Images captured of the adipocytes from the florescent inverted microscope of one pregnant woman is presented in the figure 1. There was no significant difference in the paired t-test values of the mean adipocyte cell area (Table 1). The spearman’s correlation (Table 1) of the different AT depots provides evidence that the two independent parameters have a strong linear relationship.

Adipocyte size determination
Scatter plots depicted in figure 2 describe the variability of the paired measurement through the ranges of measure. Bland Altman plot assessed the agreement, displaying the mean difference between the H & E stained AT and collagenase digested isolated adipocytes (Figure 3). Most of the values (97.48% of the total adipocytes) were within the 95% limits of agreement (Table 1). The adipocytes with larger size have a tendency to become overestimated in all AT depots.

The cell viability was calculated as described. The percentage viability of the adipocytes at both time points (immediately and 3 hours after isolation) was found to be 100%.

The adipocyte size was compared in two groups of pregnant women (3 normal weight pregnant women and 3 overweight pregnant women) (Table 2). When the adipocyte size was considered the adipocyte size was significantly higher in the sSAT of overweight pregnant women (4720.6±370.2) than the normal weight pregnant women (2630.9±324.9)(P=0.006). Furthermore, dSAT of overweight pregnant women (4336.2±699.4) was also significantly higher when compared to normal weight pregnant women (2013.3±562.1)(P=0.03).

**Discussion**

This study demonstrated that the isolated adipocytes can be used as a cost effective simpler and accurate method for the cell size determination. The two methods were strongly correlated in all adipose tissue depots. As analyzed by Bland-Altman plot, 97.48% values were within the limit of agreement, therefore the two methods were found to be bias. In a similar study performed to compare the adipocyte size in collagenase digested isolated adipocytes, stained adipocytes and Osmium fixed adipocytes, the techniques were found to be strongly intercorrelated (10). They have found that

![Figure 1 Collagenase digested isolated adipocytes of (A) superficial subcutaneous adipose tissue, (B) deep subcutaneous adipose tissue and (C) visceral adipose tissue. H & E stained adipose tissue of (D) superficial subcutaneous adipose tissue, (E) deep subcutaneous adipose tissue and (F) visceral adipose tissue of one pregnant woman.](image)

Table 1: Comparison of the two methods. sSAT: superficial subcutaneous adipose tissue, dSAT: deep subcutaneous adipose tissue, VAT: visceral adipose tissue.

<table>
<thead>
<tr>
<th></th>
<th>Size of adipocytes (Mean ± SD) µm²</th>
<th>Student’s paired t test</th>
<th>Spearman’s correlation</th>
<th>Bland Altman</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>H &amp; E stained adipocytes</td>
<td>Isolated adipocytes</td>
<td>r</td>
<td>p value</td>
</tr>
<tr>
<td>sSAT</td>
<td>3843.46±5286</td>
<td>3140.93±6172</td>
<td>0.337</td>
<td>0.97</td>
</tr>
<tr>
<td>dSAT</td>
<td>3020.43±1957</td>
<td>2426.16±5420</td>
<td>0.615</td>
<td>0.99</td>
</tr>
<tr>
<td>VAT</td>
<td>3189.86±2515</td>
<td>2140.5±5095</td>
<td>0.794</td>
<td>0.99</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td></td>
<td>0.582</td>
<td>0.99</td>
</tr>
</tbody>
</table>
Osmium fixed adipocytes have a strong association with cardio metabolic risk factors, however this is an expensive technique and also it was stated by them that Osmium fixed adipocytes are larger due to the space filled by osmium during fixation.

Isolation of adipocytes can be performed using basic laboratory equipment and is less time consuming (10, 21), while histological sample preparation is more time consuming (19) and require skilled personnel.

The demerits caused by collagenase digested isolated adipocytes were addressed in our study as follows. We excluded larger cells and the cells between the size of 150-50,000μm² taken for our calculation. This exclusion of cells also results in exclusion of adipocytes which had lost the actual shape and size during the isolation process. In ImageJ analysis, the distorted cells were excluded. Since previous studies showed that, some larger collagenase digested isolated adipocytes tend to be distorted due to collagenase treatment and these changes in the morphology may affect the results (4). Moreover in the present study the images of adipocytes were captured as soon as they were isolated and a cover slip was placed over the cell suspension. Prolonged storage of the collagenase digested isolated adipocytes suspension cause changes in the morphology in the adipocytes (3) and the collagenase digested isolated adipocytes are unfixed therefore they may be floating and the same adipocyte may be captured more than once (4). In our study the cell viability was assessed and the cells were found to be viable throughout

Adipocyte size determination

Fig. 2. (A) Correlation between isolated and H&E stained adipocytes in superficial subcutaneous adipose tissue (n=450) (B) Correlation between isolated and H&E stained adipocytes in deep subcutaneous adipose tissue (n=450) (C) Correlation between isolated and H&E stained adipocytes in visceral adipose tissue (n=450). sSAT: superficial subcutaneous adipose tissue, dSAT: deep subcutaneous adipose tissue, VAT: visceral adipose tissue.
Fig. 3. Bland-Altman plots for the comparison of adipocyte size in isolated and H&E stained adipocytes in (A) superficial subcutaneous adipose tissue, (B) deep subcutaneous adipose tissue and (C) visceral adipose tissue (n=450 adipocytes).

Table 2: Characteristics of pregnant women of the two groups

<table>
<thead>
<tr>
<th>Pregnant women groups</th>
<th>Age (years)</th>
<th>Parity</th>
<th>Weight at first antenatal visit (Kg)</th>
<th>Height (cm)</th>
<th>Weight delivery (Kg)</th>
<th>BMI at delivery (Kg/m²)</th>
<th>Weight gained during pregnancy (Kg)</th>
<th>Weight of baby (Kg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal weight group (Mean±SE)</td>
<td>34±3</td>
<td>52.8±4.7</td>
<td>154.7±3.1</td>
<td>22.0±1.2</td>
<td>63.8±6.2</td>
<td>26.6±2.1</td>
<td>10.1±3.1</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Participant 1</td>
<td>40</td>
<td>1</td>
<td>53</td>
<td>152</td>
<td>22.94</td>
<td>68</td>
<td>29.43</td>
<td>15.0</td>
</tr>
<tr>
<td>Participant 2</td>
<td>31</td>
<td>-</td>
<td>61</td>
<td>161</td>
<td>23.55</td>
<td>72</td>
<td>27.79</td>
<td>11.0**</td>
</tr>
<tr>
<td>Participant 3</td>
<td>31</td>
<td>1</td>
<td>44.5</td>
<td>151</td>
<td>19.58</td>
<td>51.5</td>
<td>22.59</td>
<td>4.3**</td>
</tr>
<tr>
<td>Overweight group (Mean±SE)</td>
<td>33.7±1.8</td>
<td>62.7±1.8</td>
<td>148.7±2.9</td>
<td>28.4±0.4</td>
<td>73.3±1.2</td>
<td>33.29±0.8</td>
<td>10.7±0.9</td>
<td>2.6±0.1</td>
</tr>
<tr>
<td>Participant 1</td>
<td>36</td>
<td>-</td>
<td>65</td>
<td>150</td>
<td>28.88</td>
<td>74</td>
<td>32.89</td>
<td>9.0</td>
</tr>
<tr>
<td>Participant 2</td>
<td>30</td>
<td>2</td>
<td>59</td>
<td>143</td>
<td>28.70</td>
<td>71</td>
<td>34.80</td>
<td>12.3*</td>
</tr>
<tr>
<td>Participant 3</td>
<td>35</td>
<td>2</td>
<td>64</td>
<td>153</td>
<td>27.52</td>
<td>75</td>
<td>32.19</td>
<td>11.0</td>
</tr>
</tbody>
</table>

Body mass index (BMI) cutoff values: Normal weight (18.5-25 Kg/m²) and Overweight (>25 Kg/m²) (WHO, 2006)  
*Individuals who gained more than the recommended weight gain  
**Individuals who gained below the recommended weight gain (Recommended weight gain during pregnancy: Normal weight (11.2-15.9Kg) and Overweight (6.8-11.2Kg)
the experiment. Therefore, we could predict minimal morphological changes during the study procedures. However, the histological slide preparation has the ability to minimize the morphological changes and the cells overlapping when compared with isolated cells (11).

The H & E stained slides also have several drawbacks. Thick histological preparations (19) and prolonged storage in 10% formalin may cause the shrinkage of the adipocytes (3). For the present study thin (5μm) adipose tissue histological preparations and only stored the samples for 1-2 months. The 10% formalin was also replaced every 2 weeks.

In addition, the clinical significance of adipocyte size measurement has been discussed extensively and has shown to have a positive correlation with serum insulin levels and triacylglycerol levels (9, 22). Moreover, the studies of Fang et al., (2015) have found an association between adipocyte size distribution and type 2 diabetes mellitus. Hence, adipocyte size measurement and the adipocyte distribution can be utilized as a measure of the extent and the tendency for chronic disease risk such as obesity, diabetics and CVD.

In the two groups studied, the adipocyte size was significantly higher in sSAT when compared to VAT in overweight pregnant women but not in normal weight women. Furthermore, the sSAT and dSAT size was significantly higher in overweight when compared to normal weight pregnant women. These findings imply the varied contribution of different adipose tissue depots in disease risk among overweight women and highlight the importance of further metabolic studies which may shed light on innovative intervention strategies in chronic disease prevention.

Conclusion
In conclusion, the collagenase digested isolated adipocytes can be used as an accurate and less cumbersome method for the cell size determination. However, the technique has demerits and merits which have to be realized by the researcher and necessary precautions needs to be taken as relevant to the study of interest.

Acknowledgement
University grant AP/3/2/2016/SG/14 and MSc vote Department of Biochemistry and Molecular Biology, Faculty of medicine, University of Colombo. The authors acknowledge the contribution of the Dr. Gihan, surgeons, and nurses of De Soysa maternity hospital. The authors also thank the study participants.

References
8. Kenéz, Á.,Kulcsár, A., Kluge, F., Benbelkacem, I., Hansen, K., Locher, L., Adipocyte size determination


