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The use of quantitative and qualitative approach to evaluate fat distribution and morphology in white adipose tissue

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ABSTRACT

Introduction: White adipose tissue (WAT) is the main responsible for the development of obesity and a diverse number of morphological techniques can be applied in its studies. Material and Methods: The present work was approved by an Ethics Committee (CEUA-827). Male C57BL/6 mice (12-wk-old) were fed a standard chow (CON) or high-fat diet (FAT) for 12 weeks. Body mass (BM) was assessed at the beginning and end of experiment period. After sacrifice and tissue extraction, fat distribution and adiposity index were measured. Plasmatic analyses were performed for cholesterol and triacylglycerol. Formalin-fixed epididymal adipose tissue was prepared for morphometric estimation of adipocytes diameter and stereological estimation of adipocytes density and cross-sectional area. Results: FAT group presented increased final BM, cholesterol, triacylglycerol and adiposity index when compared to CON group. Fat distribution revealed an increased amount of retroperitoneal, epididymal and inguinal fat pads in FAT in comparison to CON group. Qualitative histological analyses showed crown-like structures in FAT group. Morphometric and stereological analyses revealed adipocytes hypertrophy with increased diameter and sectional area in FAT group when compared to CON. FAT group also presented decreased numerical density of adipocytes in comparison to CON. Conclusion: The methods used to analyze WAT in this study act as important tools to elucidate morphological differences in this tissue induced by high fat diet in an obesity model.

Keywords: white adipose tissue (WAT), high-fat diet (HF), stereology, morphometry

INTRODUCTION

White adipose tissue (WAT) is a type of connective tissue specialized in lipid storage with a wide range of functions through the body. In addition to energy storage function, WAT acts in immune system, local tissue architecture and also has an endocrine role secreting a wide range of factors known as adipokines [1]. Morphologically, WAT's adipocytes contain one single droplet of lipids that pushes the cytoplasm and nucleus to the cell periphery and its location is divided into subcutaneous (beneath the skin) and visceral (around internal organs). The large gluteofemoral subcutaneous depot in humans can be equivalent to the inguinal fat pad in rodents, in terms of location. Moreover, rodents accumulate visceral fat pads in the perigonadal region, known as epididymal in males, as well as retroperitoneal fat pads connected to the posterior abdominal wall near the kidneys and the abdominal portion of the ureters [2].

Visceral WAT or intra-abdominal fat pad expansion is more associated to development of

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metabolic disorders [3]. WAT is the main responsible for the evolution of obesity and metabolic syndrome through the instauration of a low-grade chronic inflammatory state that leads to all the metabolic abnormalities found in these pathological states [1]. The development of inflammation in WAT is associated to crown-like structures. These arrangements of cells are formed by an accumulation of macrophage surrounding the WAT and are commonly found in obese conditions [4].

Numerous studies use morphological methods as tools to evaluate WAT parameters. Some methods of studying WAT in literature include fat distribution, to compare the proportions of subcutaneous and visceral fat [5] and the adiposity index, used to measure the percentage of body fat in comparison to body weight [6]. Morphometry and Stereology are other widely used methods to WAT studying [7]. Some of the commonly evaluated measures are the mean diameter and numerical density of adipocytes [8, 9] and their cross-sectional area [10].

High-fat diet is a widely used diet-induced obesity model capable of afflicting morphological alterations in WAT [11]. Therefore, in this study we sought to assemble morphological techniques used in the analyses of WAT, comparing their use in a high-fat diet model with a control diet.

MATERIAL AND METHODS

Animals and diet

The experimental protocols of this study were approved by the local committee for animal experimentation at the Fluminense Federal University (Protocol Number CEUA - 827). Animal care and procedures were carried out in strict accordance with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH guide, 8th edition, 2011). The animals were housed in a room with controlled temperature (22 ± 2°C), 12h/12h dark-light cycles and free access to food and water.

Male C57BL/6 mice (12 weeks old) were randomly divided into two nutritional groups: standard chow diet group (SC; 14% protein, 10% fat, and 76% carbohydrates, total energy 15 kJ/g; n=10), and high-fat diet group (FAT; 14% protein, 50% fat and 36% carbohydrates, total energy 21 kJ/g; n=10). The diets were manufactured in accordance with the recommendations of the American Institute of Nutrition (AIN-93M) [12] and the mice were fed for 12 weeks.

Body mass of animals was measured at the beginning and end of the experimental period and its values are presented as initial and final body mass.

Sacrifice and Tissue Extraction

After 12 weeks of diet, the animals were deprived of food for 8 h and were deeply anesthetized (intraperitoneal ketamine, 40 mg/kg, and xylazine, 8 mg/kg). Blood samples were rapidly obtained via cardiac puncture and then the rats were killed by exsanguination. The fasting plasma was separated via centrifugation (120 g for 15 min) at room temperature, and stored at -20ºC until further analyses could be performed.

Inguinal fat pad (subcutaneous fat located between the lower part of the rib cage and the mid-thigh) and the intra-abdominal fat pad (a retroperitoneal fat connected to the posterior abdominal wall near the kidneys plus genital fat located in the lower part of the abdomen and connected to the epididymis in males) were carefully dissected and weighed. The epididymal fat pads were kept in freshly prepared fixative (formaldehyde 4% w/v, 0.1 M phosphate buffer pH 7.2) for 48 h and prepared for light microscopy. The adiposity index was defined as the ratio between the sum of the intra-abdominal and subcutaneous masses divided by the total body mass, presented as a percentage.

Plasma Analysis

The concentrations of plasma total Cholesterol and Triacylglycerol (TAG) were performed using a colorimetric enzymatic assay kit and measured with an automated spectrophotometer (Synergy H1, Hybrid Multi-Mode Reader, BioTek Instruments, VT, USA).

Adipocyte microscopy

The formalin-fixed epididymal adipose tissue was embedded in paraffin and 5-μm-thick sections were stained with Hematoxylin & Eosin. Digital images were acquired randomly (Leica microscope DM500, Wetzlar, Germany). Ten digital images per animal were studied to determine the morphometric and stereological estimations [7].

The diameter of at least 50 adipocytes per animal was measured using the Image-Pro Plus software (Media Cybernetics, Silver Spring, MD, USA). The adipocytes were randomly chosen for quantification and the results were used to establish the distribution of adipocytes in three groups: small (mean diameter < 35μm), medium (mean diameter < 70μm and ≥ 36μm) and large (mean diameter ≥ 71μm) [8].

The numerical density of adipocytes was measured in a frame of known area produced by the STEPanizer web-based software (www.stepanizer.com) [9].

Furthermore, the cross-sectional area of the adipocytes was defined as the ratio between volume density of adipocytes, measured by a 36-point test-system produced by the STEPanizer software, and twice the numerical density of adipocyte per area as previously described [10].

Statistical analysis

Data are presented as means ± SEM. Differences between groups were tested by parametric Student t Test. In all cases, $P < 0.05$ was considered statistically significant. Analyses were performed using GraphPad Prism version 6.02.

RESULTS

Initially, body mass showed no difference between groups. After 12 weeks of high fat diet intake, the FAT group increased the body mass when compared with CON (+25.01%, $p < 0.001$) groups (Table 1).

Lipid profiles were assessed to evaluate the detrimental effects of the experimental diet and can be observed in Table 1. Cholesterol and TAG levels were increased on FAT (CHO: FAT, +45.68%, $p < 0.0001$; TAG: FAT, +70.28%, $p < 0.0001$) group in relation to the CON group.

Our results demonstrate that high-fat diet intake influences fat pad distribution (Figure 1). The amount of retroperitoneal fat was increased in the FAT group when compared with CON (+244.58%, $p < 0.0001$) group. Similar results were observed when concerning epididymal fat. The FAT group had larger epididymal fat pad than CON (+251.77%, $p < 0.0001$) group. Furthermore, the inguinal fat depot mass was also increased in the FAT group when compared with CON (+254.02%, $p < 0.0001$) group. In addition to this, the FAT group had increased adiposity index (+78.66%, $p < 0,001$) when compared with CON group (Figure 1).

Histological analysis of stained sections from epididymal adipose tissue showed an enlargement of adipocytes and also revealed collagen deposition in the FAT group. Following increased fat pad size, adipocyte hypertrophy and fibrosis, crown-like structures were observed in the FAT group (Figure 2A), suggesting macrophage infiltration surrounding dead or dying adipocytes.

The stereological analysis of epididymal adipose tissue showed adipocyte hypertrophy in FAT group when compared with CON (+61.91%, $p < 0.0001$) group (Figure 2B). This hypertrophy is also evidenced by increased adipocyte sectional area in the FAT group (+113.82%, $p < 0.0001$) in relation to the CON group, as shown in Figure 2E.

Considering the adipocytes diameter range (Figure 2C), small adipocytes (diameter smaller than 35 μm) were more present in the CON group when compared with FAT (+89.65%, $p < 0.01$) group. Mean adipocytes were considered those with diameter between 36 and 70 μm. No differences on the percentual of mean adipocytes were observed between groups. Large adipocytes (diameter bigger than 71 μm) were predominant in the FAT group when compared with CON (>1000%, $p < 0.0001$) group.

The FAT group also had lower numerical density of adipocytes per area in relation to CON (-52.60%, $p < 0.0001$) group (Figure 2D).

Table 1: Body mass (n=7) and lipid metabolism (n=5) data. Values represent means ± SEM.

The unpaired Student's t-test (P<0.05), when compared with the CON group [a].

Data	CON	FAT
Initial Body Mass (g)	25.8 ± 0.59	25.0 ± 0.72
Final Body Mass (g)	29.8 ± 1.25	37.2 ± 0.71[a]
Plasma Total Cholesterol (mg/dL)	114.7 ± 4.62	167.1 ± 6.86[a]
Plasma Total Triacylglycerol (mg/dL)	129.8 ± 1.33	150.7 ± 1.34[a]

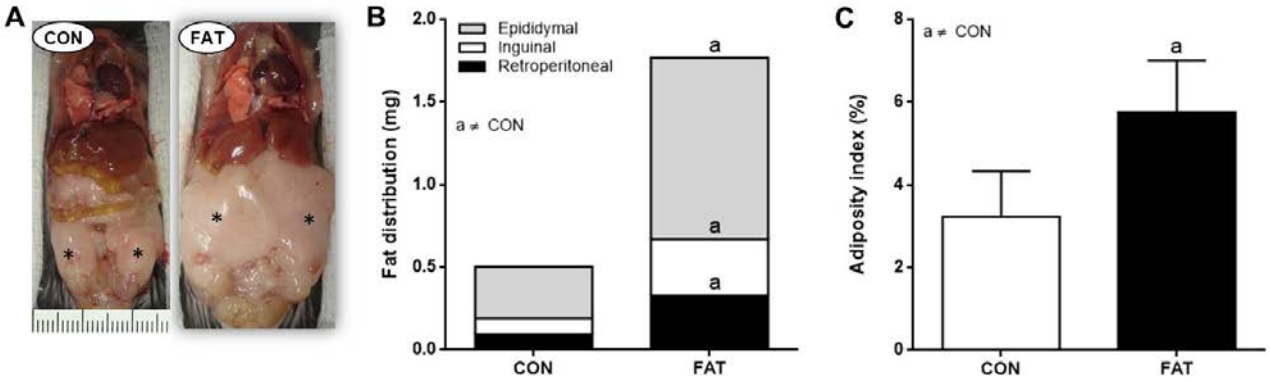

Figure 1: Fat pad distribution. (A) Chest and abdominal cavity ventral view of dissected mice showing the epididymal fat (asterisks). In FAT group, the fat pads are greater than in CON group. (B) Fat (epididymal, retroperitoneal and subcutaneous) pads distribution and (C) adiposity index analyzed by the unpaired Student's t-test. Values are shown as means (n=7). Symbol represent a significant difference ($p < 0.05$) compared with the CON group [a].

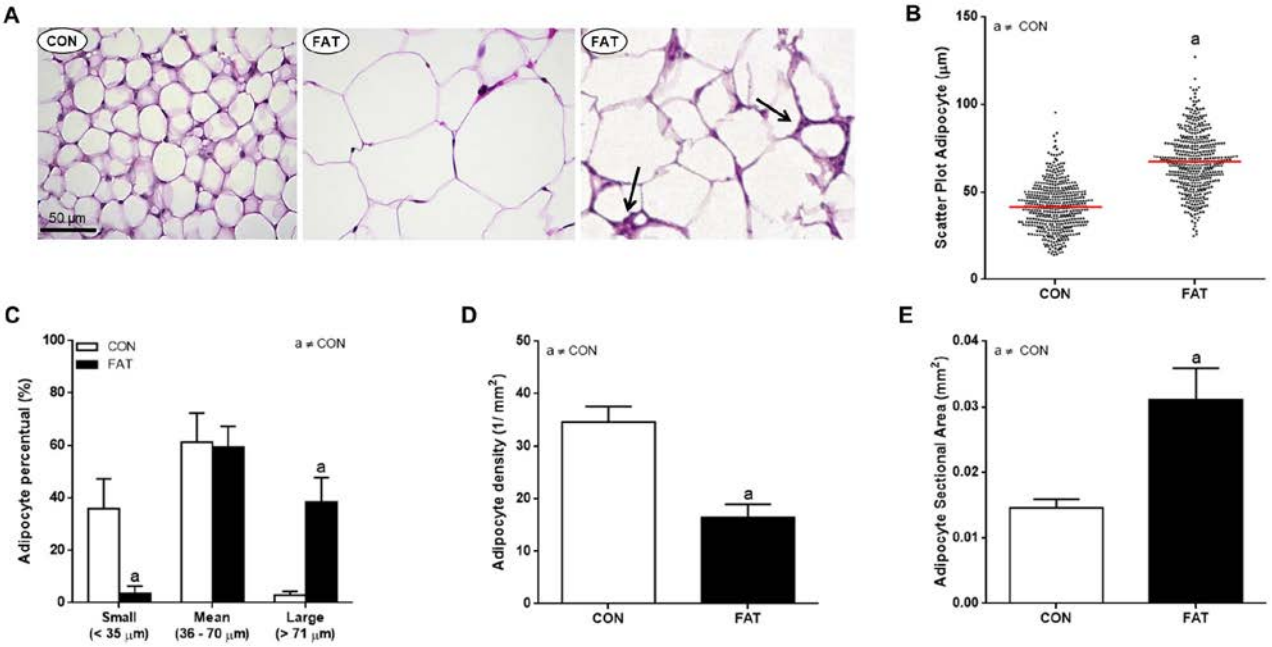

Figure 2: Adipocyte morphometry. (A) Representative light micrographs of Epididymal adipose tissue sections (stain, hematoxylin and eosin; same magnification, bar = 50 µm) showing normal white adipose tissues in the CON group; FAT adipocyte sizes were considerably hypertrophied in comparison with CON group; and the crown-like structures (arrows) were observed in the FAT group. (B) Adipocyte diameter distribution (bars represent the median). (C) Adipocyte size distribution in small (<35 µm), mean (36 – 70 µm) and large (>71 µm). (D) Numerical density of adipocytes per area (QA [adipocytes]/mm2). (E) Adipocyte sectional area. Data are reported as the means ± SEM, n=6; $p<0.05$, the unpaired Student's t-**test: [a] ≠ CON group.**

Accumulation of visceral fat is a key phenomenon in the onset of obesity-associated metabolic disorders. The present study induced obesity to evaluate quantification methods in WAT. The main findings were increased body mass, hypercholesterolemia, hypertriglyceridemia, increased amount of retroperitoneal, epididymal and inguinal fat pads, increased adiposity index, adipocyte hypertrophy, reduced adipocyte density and higher frequency of large adipocytes in the FAT group.

It is well established that high-fat diet induces obesity, dyslipidemia and increased adipose tissue mass [13, 14]. The assessment of plasma cholesterol and TAG is well described in the literature for the evaluation of dyslipidemia [15, 16]. Herein, the intake of a high-fat diet led to increased plasma levels of cholesterol and TAG.

The adipose tissue and its distribution are classically related to metabolic disorders such as cardiovascular diseases and type 2 diabetes mellitus [17]. There are several fat depots that vary in structural organization, cellular size, and biological function and since some are more related to disease development, the quantity of each depot is an important metabolic measure [17, 18]. The most relevant fat depots are epididymal, retroperitoneal and inguinal fat pads [17]. In this study, the amount of all three fat pads was increased due to a high-fat diet intake, but the epididymal fat depot showed the higher amount. Similar results were observed in other studies. After 4 weeks of high-fat carbohydrate-free diet intake, Cani et al. observed increased amount of all fat depots, although inguinal fat pad had the highest percentual in relation to body weight [19]. In the meantime, other study showed that offspring fed with a high-fat diet for 9 weeks (with control diet fed dams) had alterations in adipose tissue distribution, with significant increase of retroperitoneal fat depot [20].

The assessment of obesity is commonly described by longitudinal weight data, but it does not consider body tissue composition. Since obesity is defined as overweight due to excess body fat, the evaluation of adipose tissue accumulation is primordial [21]. The dissection and weighing of fat depots also provide the adiposity index, which translates as the percentage of fat in the body weight [21, 22]. Herein, not only the FAT group had increased body mass but also showed increased adiposity index, hence confirming the diet induced obesity after 12 weeks of high-fat diet intake. Other studies showed increased adiposity index after 32 days [23], 4 and 6 months [9].

The epididymal fat depots are considered a risk factor for the development of metabolic disorders and have greater lipogenic, lipolytic and inflammatory activity. It also represents a major contribution to plasma free fatty acid levels and thus are an important link between obesity and the development of dyslipidemia [17, 24].

Following its relation to body composition and fat distribution, adipocyte size represents an important biomarker of metabolic dysfunction related to obesity. The hypertrophy of adipose cells leads to stimulated lipid metabolism, impaired glucose metabolism and inflammation [3]. Several methods have been described to estimate adipocyte size, the most commonly used being collagenase digestion, osmium tetroxide fixation and histological analysis. Each technique has its limitations. Collagenase digestion presents the risk of damaging or excluding certain cells, and even requires specialized labor and expensive equipment. Osmium tetroxide fixation has the drawbacks of reagent toxicity and can also lead to cell swelling [3, 25].

Although histological analysis requires assumption of tissue uniformity, it is the only technique that allows examination of global tissue architecture and it is also less complex, time consuming and expensive [3]. Routine hematoxylin-eosin stained slides are a useful tool since it can reveal alterations in tissue composition and morphology, such as changes in adipocyte size and formation of crown-like structures [26]. In obese animals and humans, WAT inflammation results from the death of hypertrophic adipocytes; macrophages infiltrating the adipose tissue of obese are arranged around dead adipocytes, forming characteristic crown-like structures [27].

Images of hematoxylin-eosin stained slides acquired from light microscopy can easily translate as adipocyte size data through morphometric analysis [28]. There are specific software technologies that can quantify adipocyte diameter,

density and sectional area. Considering hypertrophy, adipocyte diameter and sectional area express the cell enlargement due to metabolic alterations [7, 28]. Since adipocyte hypertrophy has been related to inflammation, altered adipokines secretion, impaired adipogenesis, ectopic fat accumulation and impaired lipid and glucose metabolism, this information is described in several studies concerning adipose tissue metabolism [3, 9]. In this study, both adipocyte diameter and sectional area were increased after the high-fat diet intake, confirming adipocyte hypertrophy. Since adipocyte density is the number of adipocytes in a determined area, reduced adipocyte density is expected in a hypertrophy scenario [9, 19, 28]. As fat quantity is not considered in this analyze, it is not possible to predict hyperplasia data. Herein, corroborating the adipocyte size data, animals with high-fat diet intake showed reduced adipocyte density.

When concerning adipocyte size range, it is believed that larger adipocytes have higher rates of lipid uptake, inflammatory cytokines release and other metabolic damages [3]. Herein, almost 40% of adipocytes from animals fed a high fat diet showed diameter larger than 71 µm. A study has shown that adipocyte diameter increases continuously throughout the feeding period and at 12 weeks of high-fat diet intake, the maximal peak diameter was 70-80 µm [29]. Other study presented nearly 30% of adipocytes from animals fed a high-fat diet with diameter close to 80 µm [30]. Considering the relation between cell size and metabolic alterations, stratifying the diameter range of adipocytes is an important tool in assessing the metabolic effects of a high-fat diet and can be achieved with morphometric data [3].

CONCLUSION

In conclusion, the methods to quantifying WAT presented in this study provide important tools to analyze morphology of this tissue and are useful to compare models of diet-induced obesity.

CONFLICTS OF INTEREST

The authors declare no conflicts of interest.

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RESUMO

Uso de abordagem quantitativa e qualitativa para avaliação de distribuição de gordura e morfologia no tecido adiposo branco

Introdução: O tecido adiposo branco (TAB) é o principal responsável pelo desenvolvimento de obesidade e diversas técnicas morfológicas podem ser aplicadas para seu estudo. Material e Métodos: O presente trabalho obteve aprovação em comitê de ética animal (CEUA-827). Camundongos C57BL/6 machos (12 semanas) foram alimentados com uma dieta controle (CON) ou uma dieta hiperlipídica (FAT) por 12 semanas. Massa corporal (BM) foi medida ao início e fim do período de experimento. Após sacrifício e extração dos tecidos, distribuição de gordura e índice de adiposidade foram mensurados. Análises plasmáticas foram feitas para Colesterol e Triacilglicerol. Tecido adiposo epididimal fixado com formalina foi preparado para análise morfométrica do diâmetro de adipócitos e estimação estereológica da densidade de adipócitos e área seccional média. Resultados: O grupo FAT apresentou aumento em BM, colesterol, triacilglicerol e índice de adiposidade quando comparado ao CON. Distribuição de gordura mostrou aumento nas quantidades de gordura retroperitoneal, epididimal e inguinal no grupo FAT em comparação ao CON. Análise histológica qualitativa mostraram a presença de adipócitos em forma de coroas no grupo FAT. Análises morfométricas e estereológicas evidenciaram hipertrofia dos adipócitos com aumento no diâmetro a na área seccional média no grupo FAT quando comparado ao CON. Grupo FAT também apresentou uma redução na densidade numérica de adipócitos em comparação ao CON. Conclusão: Os métodos usados para analisar o TAB neste estudo se mostram importantes ferramentas na elucidação de diferenças morfológicas neste tecido em modelo de obesidade induzida por consumo de dieta hiperlipídica.

Palavras-chave: tecido adiposo branco, dieta hiperlipídica, estereologia, morfometria