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METHODOLOGY OF ADIPOSE TISSUE TYPE DETECTION IN MAMMALS

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Abstract
Nowadays, an interest in studying the composition, properties and functions of adipose tissue (AT) is growing among researchers, which is conditioned by its important role in the normal functioning of the body. Due to different types of adipose tissue (AT) in mammals (white, beige, brown and pink) and different physiological tasks performed by each type of AT, rapid, correct and effective detection of an AT type is highly topical. Methods used today are labor consuming and in the case of NMR and CT expensive, which limits possibilities of scientists. In this connection, the aim of this research was to develop a methodological approach allowing rapid and effective detection of an adipose tissue type. A methodology was formed based on the concept, formalized requirements for the method, step-wise structure of investigations and interpretation of results. The concept is based on differences in the structure of the adipose cell (adipocyte) of different AT types. The method is based on extraction of heme containing proteins. To this end, solvents and parameters of extraction that facilitate their better extraction have been chosen. An AT type has been determined by the total content of iron contained in the cytochrome fragment. Our own modification was selected. This modification includes preliminary mincing of a sample with the ice-cold TES buffer (pH 8.5) in a ratio of 1:5 (g: mL), homogenization at 9,000 rpm for 2 min with the following centrifugation at 10,000 g and 4 °C for 15 min. Effectiveness of the proposed method was confirmed by the histological and electrophoretic analyses. Therefore, the new methodology of identification and differentiation of adipocytes was proposed for rapid and effective detection of an adipose tissue type.

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Introduction

Adipose tissue (AT) is a type of connective tissue, which cells are filled with a fat droplet [1]. Adipose tissue is distributed throughout the whole body and plays a key role in energy homeostasis of the body as a reservoir of lipids. In addition, adipose tissue produces and releases various pro-inflammatory and anti-inflammatory factors, including adipokines (leptin, adiponectin, resistin and visfatin), as well as cytokines and chemokines, monocyte chemoattractant protein-1 and others [2]. Distribution of adipose tissue in mammals depends on genetic and ecological factors, while its lipid composition strongly depends on a biological species, diet, climate, and so on.

Historically, mammalian adipose tissue was divided into two types: white adipose tissue (WAT) and brown adipose tissue (BAT) based on their visible difference in color as well as their different physiological functions. WAT represents an overwhelming majority of AT in the body. It consists of many types of cells, but adipocytes are prevalent. White AT is a place of energy storage, while the main role of brown adipose tissue is thermogenesis, especially in small mammals and newborn humans [2]. Anatomically WAT is present in two main depots: subcutaneous and visceral around internal organs [3,4]. White adipose tissue specializes in processing fatty acids and triglycerides, and is critically important for energy storage, endocrine communication and insulin sensitivity [4,5]. The other function of WAT is mechanical protection of muscles and internal organs, as well as maintenance of the body temperature [6]. For example, WAT acts as a shock absorber, ensuring padding at different anatomical sites, while omental WAT is one of the visceral depots of adipose tissue, surrounding and protecting inner organs from physical injuries [7]. WAT is also known as an endocrine organ, especially visceral AT producing adipokines, which take part in metabolism or transport of lipids, immune system, blood pressure regulation, blood coagulability, glycemia homeostasis, angiogenesis, and so on [6, 8].

Brown AT is the main place of thermoregulatory non-shivering thermogenesis [9]. BAT plays an endocrine role releasing endocrine factors in the conditions of thermogenic activation, which affect the glucose level, insulin tolerance and sensitivity [10]. Brown AT is easily found both in infant and adult mammals; however, with aging, it is gradually replaced by WAT and an amount of BAT becomes rudimental in later life [3]. BAT contains abundant mitochondria; free fatty acids serve as substrates for lipid oxidation and potent activators of mitochondrial uncoupling protein 1 (UCP1) — the key transmembrane protein, which catalyzes heat generation at the mitochondrial level [11]. UCP1 is the only member that is capable to transfer protons through the mitochondrial inner membrane of brown adipocytes, uncouples respiration and ATP synthesis, and therefore, causes dissipation of energy as heat, and also stimulates the high level of fatty acid oxidation.

Thus, one of the main differences between white and brown AT is the mechanism of ensuring thermogenesis. BAT cells effectively oxidize glucose and fatty acids. With that, a large amount of heat is released and insignificant part of energy is accumulated as ATP (adenosine triphosphate). Also, because of the high content of mitochondria and ability to increase the cellular metabolic rate, BAT generates heat due to chemical energy [12]. White adipocytes are located throughout the whole body and store triglycerides as an energy buffer in large monolayer lipid droplets. During fasting and upon reduced insulin levels, the rate of fatty acid oxidation is doubled and WAT acts as the main energy source [13].

Unlike white adipocytes, brown adipocytes have abundant mitochondria, which cytochromes contain heme iron determining the brown color of tissue. For cytochromes, the catalytic cycle is typical, which is accompanied by transition of electrons upon a change in iron valence. The decisive role of the heme group consists in the fact that it converts atomic oxygen into the reactive form, which is responsible for all reactions occurring in the catalytic cycle.

Brown-like adipocytes can appear in WAT. They are a result of WAT "browning". Today, scientists assign this adipose tissue to a third type of AT and call it beige AT (brite — brown-white) [14–16]. It was noticed initially that beige AT appears as a response to cold exposure; however, other factors such as diet, physical activity, pre- and probiotics, pharmaceutical and plant substances and so on can cause "beigeing" or "browning" of WAT [17]. Beige AT is similar to BAT in terms of functions; it contains a large number of mitochondria and has high thermogenicity. Beige adipocytes can secrete certain factors that affect the function and systemic metabolism of WAT [18]. Beige AT plays a key role in adaptive thermogenesis [19], which depends on the presence of UCP1 [20].

It is interesting that today five types of adipocytes, five types of AT, are distinguished in the mammalian body. Besides white, beige and brown adipocytes, there are yellow adipocytes that are present in marrow and pink adipocytes revealed in tissues of the mammary gland during pregnancy and lactation [21].

General classification of adipose tissue is based on the AT color, which corresponds to the lipid content, mitochondrial density and vascularization (formation of blood vessels). White adipocytes contain one lipid droplet that occupies about 90% of the cell space. BAT is highly vascularized, and brown adipocytes contain a large number of mitochondria; the lipid droplet is smaller and is represented by multiple vacuoles. Beige adipocytes show characteristics of both brown and white adipose cells; the content of mitochondria is higher than that in white adipocytes and a lipid droplet is not single but bigger than in brown adipocytes [17].

At present, an interest in studying the composition, properties and functions of all AT types is growing among scientists, which is conditioned by an important role of adipose tissue in the normal functioning of the body and absence of diseases. For example, Price et al. [22] studied the functions of white and brown AT, as well as the role of microRNA in regulation of differentiation of these ATs. The authors established that induction of thermogenesis in BAT or WAT leads to an increase in the energy expenditure and prevents the development of obesity and metabolic dysfunction caused by diabetes. Adipose tissue, its properties, function, quantity and distribution in the body have been widely studied in medicine and the food industry.

In medicine, methods for AT detection that characterize its total content in the body, quantity and a degree of the development of the certain tissue type are used. One of the methods is X-ray densitometry based on generation of radiation with stable energy by an X-ray source. Due to attenuation of low-energy radiation, the highest energy level (R-volume) is determined and compared to the calibration curve of the known R-volumes. As a result, the density of soft tissues is assessed and the quantity of AT is determined. The most precise methods of diagnostics used in medicine are computed tomography (CT) and nuclear magnetic resonance imaging (NMR imaging). The CT method is based on the clear difference in X-radiation obtained from bone and adipose tissue as well as tissue that is free from fat. Using a computer processor, visual information about the character of ionizing X-radiation is obtained in a form of cross-sections [23]. In addition, nuclear magnetic resonance (NMR) is used as an additional method for assessing different AT types. NMR is based on interaction between charges of hydrogenic atoms (protons), which are present in all biological tissues. The other method that is also frequently used to study AT is histological analysis, which is applied mainly in the field of the technology development and production of meat and meat products to predict technological 'behavior' of adipose tissue and detect a ratio of all AT types [24].

These methods are time consuming and in the case of NMR and CT expensive, which restricts researchers in terms of rapid and available detection of an AT type. The aim of this research was to develop a methodological approach allowing effective detection of an adipose tissue type within a short time. The proposed method was based on the selection of solvents and extraction parameters that allow extraction and detection of the concentration of total iron contained in the fragments of cytochromes, which presence in a large quantity is characteristic of brown adipose tissue.

Objects and methods

The objects of the research were AT samples: subcutaneous and visceral white AT (WAT) and interscapular brown AT (BAT) from laboratory Wistar rats at the age of 6 or 18 months obtained from the nursery for laboratory animals "Rappolovo" of the Ministry of Science and Higher Education of the RF (Vsevolozhsky district, Leningrad Oblast, Rappolovo). Animal keeping and all manipulations with them were carried out in a compliance with Order of the Ministry of Health of the RF No. 267 of 19.06.2003 "On approval of rules of laboratory practice" and Council Directive 86/609/EEC. The experiment was approved by the bioethical commission of the V. M. Gorbatov Federal Research Center for Food Systems of RAS (protocol No. 01/2019 of 09.05.2019).

To develop the methodology, solvents and parameters of extraction were selected that facilitated better extraction of heme containing proteins, which was assessed using measurement of total amount of iron. The correctness of the proposed method was confirmed by the histological and electrophoretic analyses of AT and extracts, respectively.

Extraction of adipose tissue

The obtained adipose tissue samples were washed in the phosphate buffered saline (PBS10X, ChemCruz, USA), sponged up with filter paper and then minced into small pieces using surgical scissors. The extractants used in this study were: 1%, 2.5%, 5% and 10% acetic acid, 0.3 M acetate buffer (pH 3.6), TES buffer (pH 8.5), containing 30 mmol/L Tris (AppliChem, Germany), 1 mmol/L EDTA (AppliChem, Germany) and 0.25 mol/L saccharose and 2% protease inhibitors (Iniprol, France). The minced AT samples were mixed with the extractant in a ratio of 1:5 (g: mL) in the case of the acetate buffer, TES buffer, and 1:5 and 1:10 (g: mL) in the case of acetic acid. Extraction of target substances with the acetate buffer and acetic acid was carried out by homogenization using a portable homogenizer S10 (Stegler, China) at 8,000 rpm for 1–2 min and then at 9,000 rpm for 2–3 min. The obtained homogenates were centrifuged at 4 °C and 14,000 g for 5, 10 or 15 min (Eppendorf, Germany).

Extraction of proteins from adipose tissue with the TES buffer was performed according to the method [25] or with slight changes. Minced adipose tissue (~ 150 mg) was mixed with the ice-cold TES buffer (700 µL) (pH 8.5). Then the mixture was stirred in cold conditions at 2,800 rpm for 1 h obtaining the extract "TES-1" or homogenized at 9,000 rpm ("TES-2"). Homogenates were centrifuged at 10,000 rpm and 4 °C for 15 min.

Determination of the total iron concentration

The concentration of total iron was determined in all fresh extracts on a biochemistry analyzer BioChem FC-360 (HTI, USA) using a ready-to-use reagent kit (HTI, USA). The results were expressed in nmol iron/g adipose tissue (nmol/g).

Electrophoretic analysis

In the protein subfraction obtained by adipose tissue extraction with the TES buffer (TES-1 and TES-2), 10% acetic acid and the acetate buffer (1 g AT per 5 mL extractant), most of extracted proteins and enzymes were studied by gel electrophoresis [26]. The protein buffer was added to the supernatant in a ratio of 1:1. To prepare the protein buffer, 1 mL 10% sodium dodecyl sulfate (SDS), 250 µL concentrated β- mercaptoethanol, 625 µL 0.5 M tris -HCl, 1.5 g urea were introduced into Eppendorf-type tubes, bromophenol blue was added until the dark color and the volume was brought to 5 mL with water. After that, the samples were heated in a boiling water bath for 5 min. The samples under study were transferred to a well of the gel in an amount of 8 µL.

To carry out vertical gel electrophoresis, a chamber "VE-10" (Helicon, Russia) was used. It was filled with 12.5% polyacrylamide gel, over which 6% gel was poured and wells were made in it to introduce samples. The solution containing 25 mM Tris-HCl, 192 mM glycine and 0.1% SDS was used as a buffer. Electrophoresis was carried out with the following parameters: the first 30 min — 60 V and then at 120 V until the dye (bromophenol blue) front reached the lower edge of the gel plates. Proteins were stained using Coomassie G-250 in the solution of the following composition: 10% acetic acid, 25% isopropanol, 0.05% Coomassie G-250. To remove unbound dye, 10% acetic acid was used.

To carry out computed densitometry, one-dimensional electropherograms in a wet state were used. Their full digital images were obtained using a Bio-5000 Plus scanner (Serva, Germany) in a mode of 600 ppi 2D-RGB. The obtained digital images of polyacrylamide gels were analyzed using ImageJ®, publicly accessible software for processing digital images developed at the National Institutes of Health (NIH, USA) [27]. Background subtraction and 8-bit format were chosen for image analysis to improve the band intensity and reveal differences between the samples under study. Within the framework of the study, protein spots were compared by optical density, and the fold change index, an excess of which by more than two units is generally considered statistically significant difference, was calculated.

Histological analysis

The fat samples were fixed in the 10% neutral buffered formalin solution (BioVitrum, Russia) for 72 hour at 22 ± 2 °C. Preparation of sections with a thickness of 12 μm was performed on a cryostat MIKROM–HM525 (Thermo Scientific, USA). The obtained sections were mounted on Menzel-Glaser slides (Thermo Scientific, USA), stained with Ehrlich's hematoxylin and 1% aqueous-alcoholic solution of eosin (BioVitrum, Russia) and embedded in glycerin-gelatin by the conventional method [28]. The histological preparations were studied using an Axio Imager A1 light microscope (Carl Zeiss, Germany) and the image analysis system AxioVision 4.7.1.0 (Carl Zeiss, Germany). No less than three sections were made for each sample. A diameter of adipocytes was measured for 100 cells in each section in the interactive mode with an accuracy of ± 0.1 μm.

Statistical analysis

In this work, the screening study for designing a method was carried out. For results, therefore, an error of measuring instruments was taken into account. Statistical data analysis was carried out using MS Excel (Microsoft, USA). The results are presented as mean ± SD. Differences were considered significant and a relationship between parameters was acknowledged at a probability level of not higher than 0.05.

Results and discussion

During the experiment, extraction of three AT samples from rats at the age of about 5 months was carried out with 10% acetic acid in a ratio of 1:5 (g: mL). Separation of extracts at the maximum rate of centrifugation (14,000 rpm) for 5 min was impossible. In this connection, the time of centrifugation was increased up to 10 min and then up to 15 min. However, an increase in time did not allow taking average sample of the supernatant of subcutaneous AT. The concentrations of total iron were determined in the samples of visceral and interscapular AT, which were 46.2 and 655.0 nmol/g, respectively.

Upon extraction of AT from animals at the age of about 18 months with 10% acetic acid in a ratio of 1:10 (g: mL), separation of all AT types into fractions was achieved. Iron concentrations (C_{Fe}) for visceral, interscapular and subcutaneous AT were 41.8 nmol/g, 105.3 nmol/g and 34.1 nmol/g, respectively. C_{Fe} for subcutaneous and visceral AT differed insignificantly, while the values for interscapular AT were 3.1 and 2.5 times higher, respectively, than the corresponding values in subcutaneous and visceral AT. It was noticed that upon extraction of interscapular AT in a ratio of 1:5 (g: mL), the iron concentration decreased by about 6.2 times compared to extraction in a ratio of 1:10 (g: mL). It can be assumed that this observation is associated with the animal age as brown tissue is gradually replaced with white AT with age, which is confirmed by Choe S. S. [3].

To check the expediency of using acetic acid, an experiment was carried out with the reduced concentration of acetic acid. Upon extraction of AT from animals at the age of about 18 months with 5%, 2.5% and 1% acetic acid in a ratio of 1:5 (g: mL), separation of homogenates of subcutaneous AT was also impossible. The iron concentration was determined in the samples of visceral and interscapular AT. The results are presented in Table 1.

Table 1. Iron concentration in AT extracts with 1%, 2.5% and 5% acetic acid

$C_{CH3COOH}$, %	C_{Fe}, nmol/g AT	
	Visceral AT	Interscapular AT
5	29.05 ± 1.45	156.55 ± 7.8
2.5	50.00 ± 2.5	144.55 ± 7.22
1	26.95 ± 1.34	207.70 ± 10.38

It is evident from Table 1 that the iron concentration in visceral AT was approximately the same when using 5% and 1% acetic acid, while with 2.5% acetic acid the iron concentration increased up to 50 nmol/g AT. In the case of interscapular AT, the maximum iron concentration was found in the extract obtained upon extraction with 1% acetic acid. It was observed that the iron concentration in interscapular AT exceeded the corresponding values in visceral tissue when using any acid concentration. For example, the iron concentration in the extracts of interscapular AT was 7.7, 2.9, 5.4 and 2.5 times higher, respectively, when using 1, 2.5, 5 and 10% acetic acid as an extractant.

During the experiment, extraction of three AT samples from rats at the age of about 18 months was performed with the 0.3 M acetate buffer in a ratio of 1:5 (g:mL). In the homogenates of all AT types, the average sample of supernatant was taken. The iron concentrations in the extracts of visceral, subcutaneous and interscapular AT were 36.55 nmol/g, 14.15 nmol/g and 79.15 nmol/g, respectively. A diagram was built (Figure 1) for visual comparison of the obtained data with the corresponding values of the iron content in the extracts obtained with the use of 10% acetic acid.

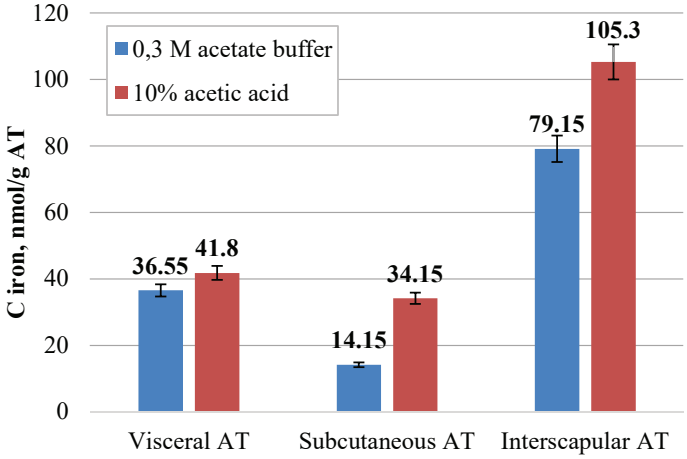


Figure 1. Content of total iron in the AT extracts, when using the acetate buffer and 10% acetic acid

It can be seen from Figure 1 that the iron content in visceral AT differed only by 5.25 nmol/g AT, when using the acetate buffer or acetic acid as an extractant, which was considered an insignificant difference. It was noted that the total iron concentration in the extracts of subcutaneous and interscapular AT obtained using the acetate buffer were lower by 19.95 and 26.15 nmol/g, respectively, compared to extraction with 10% acetic acid. The data obtained allow suggesting that the acetate buffer is a less promising extractant of heme containing proteins from adipose tissue compared to 10% acetic acid. However, its use enables more effective separation of AT extracts into fractions irrespective of the AT type and location.

Comparative analysis of extraction effectiveness of heme containing proteins from adipose tissue by the selected extraction methods was carried out. These methods included: 10% acetic acid and acetate buffer in a ratio of 1:5 (g: mL) and the use of TES buffer in a ratio of 1:5 (g: mL) without and with homogenization (TES-1 and TES-2, respectively) (Figure 2).

It is evident from Figure 2 that all selected extractants enabled taking the average sample of the supernatant. However, in the case of subcutaneous AT, the average sample was extremely small when 10% acetic acid was used, which did not allow performing a large number of experiments. It was noticed that in the case of TES-1, the upper layer was characterized by heterogeneity and the color of the extract of interscapular AT was duller compared to the color in the case of TES-2, which in turn was identical to the color obtained when using the acetate buffer. The extract of interscapular AT (BAT) with 10% acetic acid was characterized by browner color compared to other samples.

In the AT extracts obtained by the selected methods for extraction of heme containing proteins, the iron concentration was determined (Table 3) and the proteomic analysis by one-dimensional electrophoresis was carried out (Figure 3).

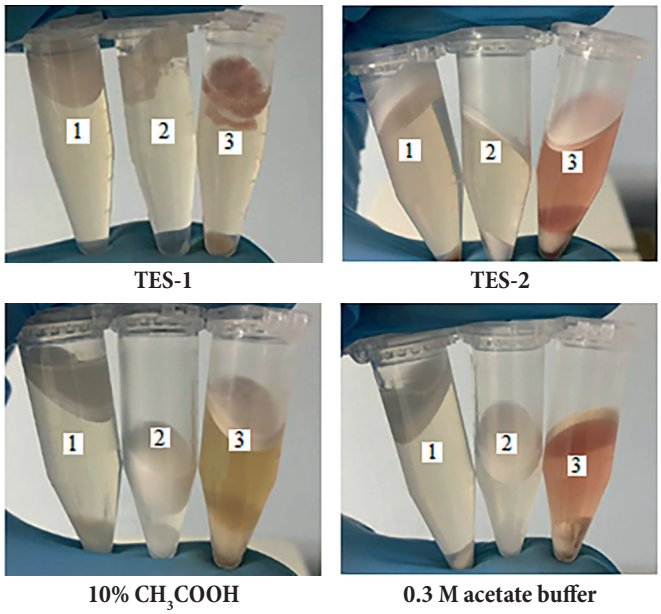


Figure 2. Results of the separation of AT homogenates using different extractants, where: **1** — visceral white AT; **2** — subcutaneous white AT; **3** — interscapular brown AT

Table 3. Iron concentration in AT extracts

AT extracts	C_{Fe}, nmol/g AT		
	Interscapular AT	Visceral AT	Subcutaneous AT
TES-1	109.15 ± 5.45	57.2 ± 2.86	59.45 ± 2.97
TES-2	675.2 ± 33.70	129.8 ± 6.49	138.95 ± 6.90
10% acetic acid	346.5 ± 17.32	105 ± 5.25	115 ± 5.75
0.3M acetate buffer	192.5 ± 9.62	24.55 ± 1.23	27.85 ± 1.39

It was noted that C_{Fe} in all types of interscapular AT extracts exceeded the corresponding values for visceral and subcutaneous AT. The iron concentrations in the extracts of visceral and subcutaneous AT significantly differed between the extractant types; however, they were close to each other. The maximum iron concentration was obtained for interscapular AT upon TES-2 extraction, and it significantly decreased in the row: TES-2, acetic acid, acetate buffer and TES-1.

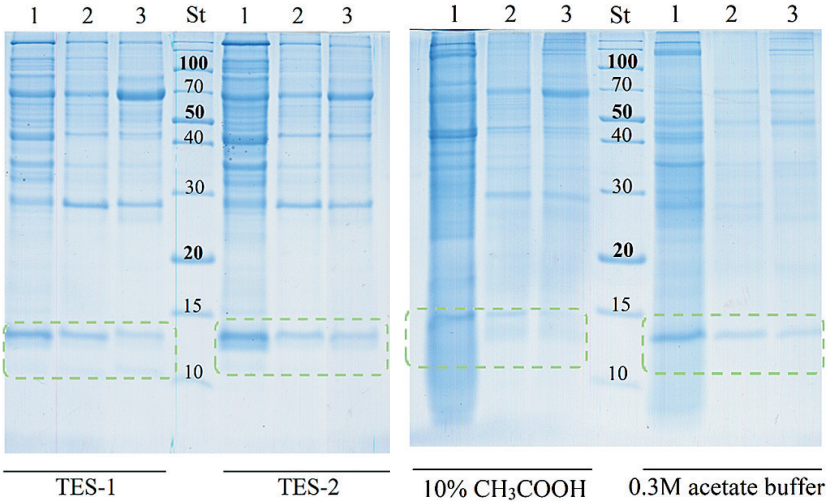


Figure 3. One-dimensional electropherogram of adipose tissue extracts, where **St** — standard of molecular weights: 250, 150, 100, 70, 50, 40, 30, 20, 15 and 10 kDa (from top to bottom), **1** — interscapular AT; **2** — visceral AT; **3** — subcutaneous AT

In the course of processing of the obtained electropherogram images, a wide spectrum of compounds in a molecular weight range from 10 kDa and higher was revealed; with that, the highest number of protein fractions was found in interscapular AT. Using the UniProt database [29], the presence of target iron containing proteins with a molecular weight of less than 15 kDa was assumed: cytochrome c, testis-specific — 11.7 kDa [30] and cytochrome c, somatic — 11.6 kDa [31]. Several significant differences in the optical density of the target protein fractions with MW from 10 to 15 kDa (marked with the green color in Figure 3) were recorded using the program complex ImageJ. The values obtained are given in Table 4. The comparative analysis in certain regions of molecular weights between different samples was carried out.

Table 4. Results of the densitometry analysis of protein fractions in the AT extracts

Samples	Optical density of target protein fraction, a.u.		
	Interscapular AT	Visceral AT	Subcutaneous AT
TES –1	3,918.4 ± 190.4	2,276.0 ± 113.1	1,247.1 ± 56.0
TES –2	5,156.0* ± 211.4	1,708.6 ± 83.3	1,119.9 ± 5.9
10% acetic acid	1,711.0 ± 61.8	947.4 ± 46.5	93.0 ± 2.2
0.3M acetate buffer	3,479.7* ± 172.6	1,308.9 ± 65.0	674.1 ± 26.8

* statistically significant difference according to Fold > 2

A large difference was revealed based on the densitometry analysis of the density of AT protein fractions. Interscapular AT was characterized by maximum values, which significantly exceeded the corresponding values for visceral and subcutaneous AT. Thus, the best variant for revealing target protein fractions with MW from 10 to 15 kDa is a variant of extraction using the TES buffer. For example, the optical density of the target fraction was 5,156.0 a.u. in interscapular AT, 1,708.6 a.u. in visceral AT and 1,119.9 a.u. in subcutaneous AT, when using extraction with the TES-2 buffer.

As a result of the performed histological investigations of the samples from rats at the age of 18 months, the photographs of the microstructure were obtained (Figure 4).

Large adipocytes with a diameter of 67.32 ± 8.13 and 75.46 ± 9.25 μm, respectively, were revealed in subcutaneous and visceral adipose tissue. The revealed adipocytes were typical of white adipose tissue and contained a single large lipid droplet and the nucleus shifted to periphery of the cell. The samples of the interscapular adipose tissue consisted of small brown adipocytes with a diameter of 20.51 ± 2.58 μm with multilocular lipid droplets inside; the nucleus was located both at the periphery of the cell and in its central part. The results obtained correspond to [32,33] regarding characteristics of white and brown adipocytes.

During the experiments, it was established that the optimal method for extraction of heme containing proteins from AT is the TES buffer with homogenization and the following centrifugation. The method makes it possible to distinguish rapidly and effectively brown AT from white AT by the concentration of heme containing proteins in it.

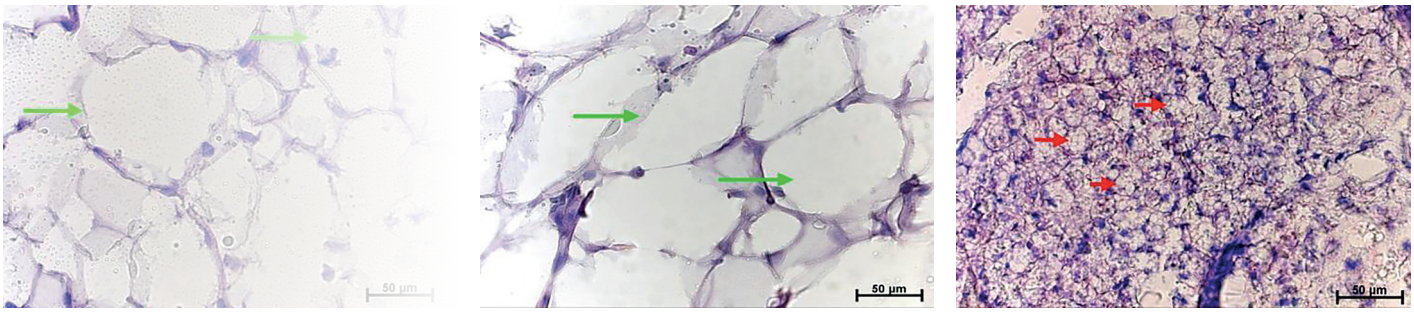
The method is easy to reproduce and it does not require special training of personnel. A biochemical blood analyzer available in a specialized laboratory is used. In future, the method can be made easier and cheaper, if necessary. In addition, a possibility of its use in identification of other AT types can be studied.

Conclusion

In the course of work, the methodology of AT identification and differentiation by types was formulated. The basis of the concept is the knowledge about the structure of adipocytes of white, brown and beige tissues, different content of iron containing proteins (cytochromes) in them as well as other information. The main requirements for the method of investigation were determined: rapidity, ease of use, minimization of hard-to-get reagents, wide accessibility of equipment being used, absence of the need for highly qualified researchers, ease of interpretation of results, possibility of metrological support.

The original investigation method has been proposed. The method consists in preliminary mincing of a sample with the ice-cold TES buffer (pH 8.5) with the protease inhibitor in a ratio of 1:5 (g/mL), homogenization at 9,000 rpm for 2 min and subsequent centrifugation at 10,000 g and 4 °C for 15 min. Certainty of detection of AT types was confirmed by the standardized histological method of analysis as well as by one-dimensional electrophoresis.

In conclusion, different AT types have been studied in detail, the effective methodology for detection of types of mammalian adipose tissue has been proposed.



Subcutaneous AT (WAT) Visceral AT (WAT) Interscapular AT (BAT)

Figure 4. Microstructure of the adipose tissue samples. Staining with hematoxylin and eosin. Scale bar 50 μm. The green arrow indicates white adipocytes, the red arrow indicates brown adipocytes

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