Ultrasonography Evaluation of Abdominal Fat in Live Rats

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Summary We have developed a new noninvasive method of estimating abdominal fat volume in live rats using ultrasonography. By this method, cross sections of perirenal (retroperitoneal) fat tissue, which is an abdominal fat, at the renal vein level could be identified and the area determined. The perirenal fat in Wistar rats (wide body weight range, 111.4 to 497.3 g; limited range, 300.1 to 337.9 g) measured by ultrasonography was compared with the actual fat tissue weight. The cross-sectional area of perirenal fat tissue was significantly correlated to the actual whole tissue weight. Using this procedure, we examined the changes of perirenal fat stores during fasting. Consequently, the cross-sectional area of perirenal fat and its actual weight decreased in parallel. Total body electrical conductivity (TOBEC) is currently used to measure fat-free mass (FFM) and indirectly predicts total body fat mass of live laboratory animals. The body fat distribution, that is, the location of adipose tissue in the abdominal region, is closely associated with obesity-related diseases. Therefore, it is important to focus not only on the accumulation of total body fat, but also on that of abdominal fat. The present ultrasonographic method is considered to be useful for repeated noninvasive measurement of abdominal fat in the live rat.

Key Words ultrasonography, abdominal fat, adipose tissue, fat accumulation, TOBEC

The measurement of body composition or fat-free mass (FFM) in live animals has recently been shown to be possible using the noninvasive total body electrical conductivity (TOBEC) method (1). This measurement principle is based on the high electrical conductivity in all lean tissues relative to fat. The noninvasive measurement of FFM in the rat was first made (2, 3) using an instrument developed for the meat industry (DjMe 100 Ground Meat Fat Tester, DICKEY-john Co., Auburn, IL,

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USA). By this method, total body fat can be indirectly calculated. The EM-SCAN instrument is claimed by its manufacturer to be able to measure FFM. The manufacturer assumes that total body water is 73.2% of FFM and incorporates this into their predictive equation. Therefore, the state of hydration of the subject is important in making an accurate FFM measurement (4, 5).

Body fat distribution, abdominal fat in particular, plays a major role in metabolic disorders, morbidity and increased mortality risk (6, 7). Therefore, it is important to observe the regional distribution of fat precisely as well as fat volume in the whole body.

Anthropometric techniques currently in use can individually measure abdominal fat tissue and subcutaneous fat tissue as well as fat volume in the whole body. Radiography, computerized tomography (CT) (8), and magnetic resonance imaging (MRI) have been used even on laboratory animals (9–11). However, they are expensive and some involve exposure to ionizing radiation. Ultrasonography is a safe, fairly inexpensive, and widely available imaging modality (12–15).

In the present study, we describe a new method for the *in vivo* estimation of abdominal fat of rats by means of diagnostic ultrasound equipment. This method was also applied to fasted rats. We compared the ultrasonography method with the TOBEC method, which evaluates FFM and indirectly predicts total body fat mass.

MATERIALS AND METHODS

Materials. Sevoflurane (SEVOFRANE) was purchased from Maruishi Seiyaku Co., Ltd. (Osaka, Japan). Ringer solution was from Otsuka Seiyaku Co., Ltd. (Tokyo, Japan). Other chemicals were obtained from Nacalai Tesque Co. (Tokyo, Japan) and Wako Pure Chemical Ind. (Osaka, Japan).

Animal treatment. Male Wistar rats (Japan Shizuoka Laboratory Center, Hamamatsu, Japan) were housed in standard cages (26 × 38 × 18 cm, three or four rats per cage). All rats were kept in an air-conditioned room at 22 ± 2°C with a humidity of approximately 50%, and a lighting schedule of 12-h light (0600 to 1800) and 12-h dark. They were provided a standard pelleted diet (MF; Oriental Yeast Co., Ltd., Tokyo, Japan), which consisted of water, 7.8; protein, 23.8; fat, 5.1; fiber, 3.2; and carbohydrate, 54.0 (g/100 g diet), and given water *ad libitum* throughout the study. The care and treatment of experimental animals conformed to the Kyoto University guidelines for the ethical treatment of laboratory animals. We performed the experiments with the least possible pain and discomfort to the animals.

Experimental design.

Experiment 1. Measurement of total body fat with TOBEC in live rats. The EM-SCAN model SA-3076 (EM-SCAN, Inc., Springfield, IL, U.S.A.), improved model SA-2, was used to estimate FFM. The rats were 4 wk old at the start of the experiment. Measurements were made at 5, 6, 7, 8, 9, 11, and 13 wk of age using

3 or 4 rats per group. The rats were fasted for 4 h for measurements which were made during the latter part of the light phase. Rats were anesthetized lightly with ether prior to measurement and then inserted headfirst into the chamber in the supine position on a plastic plate. TOBEC values (E-values) were taken within a 10–20 s period in triplicate for every rat and averaged for each measurement point. Subsequently, in the second experiment, the first trial was conducted, and then the rat's fur was thoroughly shaved off and carcasses (minus fur, food in the gut and approximately 4 to 10 mL of blood) were autoclaved for 1 h (16). Each carcass was immediately smashed with a spatula and freeze-dried about for 2 d under vacuum. The dry matter was ground to produce a uniform product in a mortar, the lipid fraction from a 1 g aliquot was extracted and the total lipids of the whole body were determined according to the method of Folch et al (17). Then, FFM was reciprocally determined. Linear regression techniques were applied to these data.

Experiment 2. Ultrasonographic measurement of abdominal fat in live rats.

Trial 1. After TOBEC measurements were performed in the first experiment, ultrasonographic measurements were conducted on the same rats; 26 male Wistar rats under continuous anesthesia with sevoflurane. Sevoflurane, an inhalation anesthetic that can easily regulate the depth of anesthesia, was chosen to minimize the damage on rats. The rats were placed in the supine position on the table, and injected intraperitoneally with approximately 5 mL/100 g body weight of a Ringer solution to obtain the clear interfaces of fat tissue on the ultrasonographic image. Next, SONO JELLY (a high-quality coupling agent for ultrasound transmission; Toshiba Co., Tokyo, Japan) was applied to the diagnostic abdomen area, and the area analyzed. The diagnostic ultrasound equipment used to evaluate the rat abdominal fat was a model SSA-220A (Toshiba Co., Tokyo, Japan) with a 7.5 MHz, 46 mm convex array probe (PVG-720S), an anthropometric technique for measurement of the skinfold depth. The long axis of the ultrasonic probe was oriented at right angles to the vertebral column, and the probe was applied vertically to the skin surface without excess compression. The operator had been thoroughly trained to be able to maintain constant pressure on the probe. The cross-sectional areas of the perirenal fat tissue were determined on the image depicting renal vein on the right and left sides, respectively (Fig. 1). The respective areas (mm²) were measured by tracing object contours on each screen using the computerized planimetric method (NIH image software; Version 1.61). The measurements were conducted three times in succession, and the average of the three measurements was determined. Rats were then sacrificed by decapitation and the adipose tissues were excised immediately. The tissues were obtained from the perirenal (retroperitoneal), epididymal, mesenteric and inguinal fat regions, and weighed.

Trial 2. For the ultrasonography measurement, all 9-wk-old rats were studied. The procedure was the same as that for the first trial. Then, the rats were sacrificed, and the perirenal fat tissues were excised and weighed.

Trial 3. The change in body fat stores during fasting was examined. Nine-week-old rats were divided into two groups, with and without 48-h fasting

Fig. 1. Cross sectional image by ultrasonographic measurement with a 7.5 MHz probe: perirenal fat tissue on the right side at the level depicting the renal vein in a live Wistar rat (263.9 g). The right picture of the same image shows the traced perirenal fat tissue contours. Image is oriented such that the vertebral column of the rat is at the right. F, perirenal fat tissue; K, kidney; S, dorsal skin; V, renal vein.

prior to the experiment. The body fat was measured after feeding for a week, the rats were sacrificed by decapitation, and the adipose tissues removed and weighed.

Statistics. The results are expressed as means $\pm$ SE. Statistical analysis was performed by linear regression and unpaired Student's *t*-test (between means with the same variance by the *F*-test). Statistics were calculated with StatView software (Macintosh Version J.4.5, Abacus Concepts, Inc., Berkeley, CA, U.S.A.). Differences with $p < 0.05$ were considered significant.

RESULTS

Experiment 1

TOBEC measurements obtained in the 26 live Wistar rats (305.1 ± 23.9 g, 5–13 wk of age) had an excellent correlation with the FFM derived from chemical analysis of the carcass (Fig. 2). While the rate of body weight gain decreased after 10 wk of age, that of total body fat indirectly predicted from FFM showed an increase at the same rate up to 13 wk of age. The body weight and total body fat values at 13 wk of age were 4 times and 14 times higher than those at 5 wk of age, respectively.

Additionally, TOBEC methodology was applied for the fasted rats in the preliminary experiment. The body fat mass theoretically calculated using the equations (Fig. 2) derived from the foregoing experiment were compared to the actual fat tissue weight in rats (control, $n=9$; 309.7 ± 3.3 g, fasted, $n=10$; 275.8 ± 4.1 g). The equations underestimated FFM and reciprocally overestimated body fat (control $7.3 \pm 0.7\%$, fasting $8.2 \pm 0.5\%$) in the fasted group.

Fig. 2. Correlation between fat-free mass (FFM) derived from chemical analysis and total body electrical conductivity (TOBEC) in 26 Wistar rats (weight range 111.4 to 497.3 g).

Fig. 3. Correlation between the cross-sectional area of perirenal fat measured by ultrasonography and the actual perirenal fat tissue weight in 26 Wistar rats (weight range 111.4 to 497.3 g). A, right-side perirenal adipose tissues; B, left-side perirenal adipose tissues.

Experiment 2

Trial 1. The cross-sectional area of perirenal fat measured by ultrasonography and the actual perirenal fat tissue weight were significantly correlated with each other over the weight range 111.4 to 497.3 g in male Wistar rats (Fig. 3). The cross-sectional area of perirenal fat ranged from 3.3 to 132.3 mm² with an average of 26.1 (± 4.9) on the right side and 31.5 (± 6.6) on the left side. The rate of fat tissue weight gain was kept constant up to 13 wk of age, while that of body weight gain was slow beyond 10 wk of age (Fig. 4). Here, the rate of gain in perirenal fat weight with time was different from that of other fat tissues. There was a marked increase in the perirenal fat weight, about 55 times, over the period of 5 to 13 wk of age. On the other hand, the weight of other fat tissues also increased about 10–20 times over the same period.

Trial 2. The same procedure as in the first trial was repeated exactly for all 9-wk-old rats ($n=32$, 319.6 ± 2.0 g) to validate the ultrasonographic method that estimates the change of perirenal fat tissue weight. The perirenal fat area ranged

Fig. 4. Change of body weight (○, broken line) and adipose tissue weights of Wistar rats at 5–13 wk of age. Adipose tissues were obtained from perirenal (●), epididymal (□), mesenteric (▲) and inguinal (△) regions. Values are means $\pm$ SE of 3–4 rats in each experimental period.

Fig. 5. Correlation between the cross-sectional area of perirenal fat measured by ultrasonography and the actual perirenal fat tissue weight in 32 Wistar rats (weight range 300.1 to 337.9 g). A, perirenal adipose tissue on right side; B, perirenal adipose tissue on left side.

from 12.4 to 53.5 mm² with averages of 24.6 ($\pm$ 1.3) on the right side and 28.6 ($\pm$ 1.6) on the left side. The correlations between the measured cross-sectional area of perirenal fat and the actual fat tissue weight of both right and left tissues were significant, although low relative to the first trial (Fig. 5). At that time, the slope of linear regression of the perirenal fat on the right side was higher than that on the left side (0.036 vs. 0.026), as in the first trial (0.084 vs. 0.066). Additionally, the weight of perirenal fat tissue on the left side was slightly greater than that on the right side in most cases (1.21 ± 0.07 g vs. 1.18 ± 0.07 g), similarly to the results in the first trial (2.05 ± 0.44 g vs. 1.94 ± 0.42 g).

Trial 3. Twelve rats at 9 wk of age were subjected to ultrasonographic measurement. After fasting treatment for 48 h, the body weight of fasted rats decreased 21 g on average, while that of control rats gained an average of 42 g (Table 1). Thus, this treatment resulted in a significant decrease in body weight (83.6%; $p < 0.0001$) and a corresponding reduction in adipose tissue weight,

Table 1. Effect of fasting for 48 h on body and adipose tissue weights of male Wistar rats.

	Control	Fasted
Body weight, g		
Initial	335.6 $\pm$ 3.7	336.7 $\pm$ 4.9 ¹⁾
Final	377.9 $\pm$ 5.5	315.9 $\pm$ 6.0**
Perirenal fat, g	3.93 $\pm$ 0.38	3.17 $\pm$ 0.55
Epididymal fat, g	4.41 $\pm$ 0.15	3.77 $\pm$ 0.37
Mesenteric fat, g	5.43 $\pm$ 0.44	3.60 $\pm$ 0.54*
Inguinal fat, g	5.09 $\pm$ 0.51	3.73 $\pm$ 0.66

Values are means $\pm$ SE of 6 rats per group.

Significant decrease from control group, * p < 0.05, ** p < 0.0001, using unpaired Student's t -test.

¹⁾ Before fasting.

Fig. 6. Variations in the cross-sectional area of perirenal fat measured by ultrasonography with changing perirenal fat tissue weight following fasting treatment for 48 h in Wistar rats ($n=6$, per group). A, perirenal adipose tissue on right side; B, perirenal adipose tissue on left side.

particularly mesenteric fat weight (3.6 g vs. 5.4 g, 66.3%; p < 0.05, Table 1). In addition, serum was assayed for free fatty acid (FFA) by an acyl-CoA synthetase and acyl-CoA oxidase enzyme method using a kit from Wako Pure Chemical Ind. (Osaka, Japan). FFA concentration significantly increased after fasting (0.917 ± 0.082 mEq/L in the fasted group and 0.323 ± 0.029 mEq/L in the control group, p < 0.0001); and this change demonstrated that FFAs were actually released from fat stores of adipose tissue by lipolysis. The values of the cross-sectional area of perirenal fat shifted in a negative direction along with the decrease of perirenal fat tissue weight in the fasted rats (Fig. 6). The perirenal fat area decreased an average of 76.6 and 81.8%, on the right and left sides, respectively, while the perirenal fat weight decreased an average of 83.4 and 78.3%, respectively.

The perirenal fat tissue was measured ultrasonographically under anesthesia

with sevoflurane weekly from age 6-wk ($n=7$, 124.5 ± 2.9 g), over a period of 3 wk, to examine the growth rate and food intake change. Consequently, the food intake declined only slightly after each measurement, and the rate of body weight gain remained constant throughout the experimental period. Namely, the stress of anesthesia with sevoflurane and ultrasonography with the intraperitoneal injection of Ringer solution was well tolerated in the rats under repeated measurements (data not shown).

DISCUSSION

Indeed, ultrasonographic measurement of the cross-sectional area of perirenal fat tissue should prove to be useful in monitoring abdominal fat accumulation in the live rat as it changes in response to a variety of experimental manipulations such as starvation or feeding of a special diet; for instance, high-fat diet or high-sucrose diet. The cross-sectional area data of perirenal fat obtained by ultrasonography significantly correlated with the actual perirenal fat tissue weight.

The TOBEC instrument used in this study, the EM-SCAN model SA-3000 series, was designed in 1994 for use with a wide variety of subject sizes. Compared to the earlier model SA-2, it has several detection chamber sizes, and gives more stable reading for growth and development of small animals (<13 g) (18). TOBEC is currently used to measure FFM and indirectly predicts the total body fat mass of live rats. Again, in the first experiment, TOBEC measurements obtained in the live Wistar rats at 5–13 wk of age correlated well with the FFM derived from chemical analysis of the carcass. Klish et al demonstrated that specific conductivity variations result from water volume, object shape, and temperature but not from solid or fat content (19). On the other hand, the estimate of FFM and body fat using TOBEC methodology has been shown to be affected by other factors such as body temperature, respiratory frequency, insensible water loss resulting from anesthesia, tail position and food deprivation (20). They pointed out that FFM or body fat should be measured immediately after the induction of anesthesia when the manufacturer's equations are used. Furthermore, it was found that the equations derived from this study underestimated FFM and reciprocally overestimated body fat of the fasted rats in the preliminary experiment. For this reason, when TOBEC measurement is applied in the study of nutrition on laboratory animals, particularly in pair-feeding experiments, great caution should be utilized to the effects of food remaining in the animal's gut on the values predicted using TOBEC methodology.

In the second experiment, the rate of perirenal fat weight gain only tended to increase with time, while the other fat tissue weight gain was kept constant over the experimental periods. These data might mean the slight differences in the characteristics of perirenal fat tissue and other fat tissues. However, West et al suggested that lipoprotein lipase activity readily increased in the perirenal and mesenteric fat tissue after the meal started, but did not change in the epididymal or dorsal subcutaneous fat tissue (21). For this reason, perirenal and mesenteric

fat tissues seem to have a common characteristic in terms of the function in the meal-to-meal (short-term) storage and release of fats.

Regional variation in adipose tissue function, cell growth and lipid storage or lipolysis has been suggested to play a role in the health consequences of some forms of obesity *in vivo* and *in vitro*. The mRNA and activity of acyl-CoA synthetase as the key enzyme of triglyceride synthesis were markedly reduced in abdominal visceral fat by exercise training (22). Abdominal adipocytes are much more lipolytically responsive to catecholamines than the subcutaneous localized adipocytes (23). This may be mainly due to enhanced functional α_2 -receptor activity and the antilipolytic effect of insulin in subcutaneous adipocytes (24), and due in part to a higher sensitivity for β_1 - and β_2 -adrenergic stimulation in abdominal cells (25). Thus, FFA are more readily mobilized from abdominal adipose tissue than from subcutaneous tissue. In this respect, mesenteric fat tissue weight decreased the most markedly by fasting in rats in this study due to a regional variation in the regulation of lipolysis. The decrease in perirenal fat tissue weight (19.3%), in contrast, was different from that of mesenteric fat tissue weight (33.7%). Nevertheless, it is thought that the cross-sectional area of perirenal fat obtained by ultrasonography could be a useful indication for noninvasive measurement of abdominal fat accumulation, not mesenteric fat.

The imaging techniques such as MRI and CT are expensive and have fairly limited availability, and ionizing radiation risks prevent them from being widely used. Simple and safe methods are needed to evaluate abdominal fat. Here, we showed that an ultrasonographic procedure can be safely repeated at frequent intervals. However, a problem and source of error of the ultrasonographic technique is that the tissue interface is not as clearly delineated as with imaging techniques (26). There are, moreover, limitations of the ultrasonographic technique; that is, only a cross section of perirenal fat is measured. Actually, this ultrasonographic method was not sensitive enough to distinguish the slight differences between the weights of perirenal fat tissues in the aged rats in the second experiment (trial 2) and those of the young rats (<7-wk-old).

In conclusion, we found that abdominal fat volume could be estimated accurately by ultrasonographic measurement of a cross-sectional area of perirenal fat. This method can be used for experimental research on fat tissue accumulation in the live rat. This ultrasonographic methodology still has limitations that need to be overcome. Additionally, training is required to avoid excess compression of the abdomen by the probe and maintain constant pressure on the probe in order to reduce reproducibility errors.

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