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Research Article

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Brown Adipose Tissue Remodeling Precedes Cardiometabolic Abnormalities Independent of Overweight in Fructose-Fed Mice

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Abstract

Background: Body fat distribution is a risk factor for several health conditions, although the literature shows that excess body fat is not always associated with cardiometabolic abnormalities in all subjects.

Objectives: To investigate glucose, lipid, and hepatic metabolism, along with white (WAT) and brown adipose tissue (BAT) remodeling in a mice model of short-term fructose feeding.

Methods: Male C57BL/6 mice (n = 10-11/group) were fed for four weeks with control diet (AIN93-M) or experimental diets rich in glucose or fructose. We investigated body weight, body adiposity, blood glucose, lipid and hepatic parameters, and WAT and BAT histopathology.

Results: Fructose feeding did not promote either weight gain or adipocyte hypertrophy of visceral and subcutaneous WAT depots, but the fat was redistributed toward visceral depots. Fructose-fed mice did not show glucose, lipid, and hepatic metabolic dysfunction, except for an elevation in total cholesterol and hepatic weight. BAT mass did not increase, and it was proportionally reduced compared with visceral WAT in fructose feed mice. BAT suffered early adverse morphological remodeling, characterized by increased lipid deposition and enlargement of intracellular lipid droplets.

Conclusion: Short-term fructose feeding redistributes fat among WAT depots, alters the ratio between BAT and visceral WAT, and promotes BAT adverse remodeling, characterized by enlarged intracellular lipid droplets.

Keywords: Obesity, Adiposity, Visceral fat, Brown adipocyte, Fructose feeding

Introduction

Obesity is strongly associated with health problems [1], however, excess body fat is not always associated with cardiometabolic abnormalities, such as insulin resistance, low-grade inflammation, and dyslipidemia. This statement came from observations that some subjects have normal weight but display excessive body fat percent and cardiometabolic dysfunction (metabolically obese normal weight, MONW), and there are also metabolically healthy but obese (MHO) subjects, that are overfat but have no signs of cardiometabolic dysfunction [2]. Regardless of body weight and body mass index, excessive body fat is associated with cardiometabolic dysfunction. The earliest signs of cardiometabolic dysfunction are excessive body fat, insulin resistance, and chronic low-grade systemic inflammation [3].

Individuals without obesity but with dyslipidemia and metabolic abnormalities have also been called as normal weight dyslipidemia (NWD) [4]. This population has an increased risk of developing nonalcoholic fatty liver disease (NAFLD), cardiovascular diseases, and type 2 diabetes [2,5]. As the liver regulates lipid metabolism and plasma lipid levels, it plays an essential role in turning an unhealthy diet and lifestyle into an unbalanced metabolic profile. The product of fasting triglycerides and glucose (TyG) is a useful index to assess both insulin resistance and hepatic steatosis/nonalcoholic steatohepatitis (NASH) in apparently healthy subjects [6,7]. Thus, it could be used as an additional early marker of metabolic dysfunction in MONW individuals.

The liver and the white adipose tissue (WAT) continuously exchange very low-density lipoproteins (VLDL) and free fatty acids (FFA), where the WAT store energy and the liver distributes it. In obesity, the liver modulates WAT inflammation and insulin sensitivity, and the hypertrophic WAT influences liver metabolism and inflammation [8]. Although WAT is a specialized lipid storage organ for excess calories, the brown adipose tissue (BAT) contains many mitochondria to dissipate chemical energy. It has been proven that BAT activity controls plasma clearance of circulating triglyceride-rich lipoproteins (TRL) by increasing its uptake into BAT and thus promoting its turnover [9].

Overall, we hypothesized that BAT morphological changes run together with cardiometabolic abnormalities independent of body weight gain. Thus, the present study aimed to investigate the early metabolic changes in glucose, lipid, and hepatic metabolism along with WAT and BAT remodeling in C57BL/6 mice by short-term fructose feeding.

Materials and Methods

Experimental design

The local Ethics Committee approved the handling and experimental protocols to Care and Use of Laboratory Animals (CEUA#647/15). The study follows the Animal Research Reporting in Vivo Experiments ARRIVE guidelines and the Guideline for the Care and Use of Laboratory Animals (US NIH Publication N° 85-23. Revised 1996) [10]. Male C57BL/6 mice at two months old were obtained from colonies maintained at the Federal Fluminense University and kept under standard conditions (12 h light/dark cycles, lights on from 7 a.m. to 7 p.m., 21±2°C, humidity 60±10%, and air exhaustion cycle 15 min/h).

At three months old, mice were randomly allocated into three groups according to the diet offered (n = 10-11/group). The control group received a purified diet, according to AIN-93M [11], and the other two groups received isoenergetic modified AIN-93M diets rich in glucose or fructose (3.81 kcal/g). Diets were obtained from Pragsolucoes (Jau, Sao Paulo, Brazil) and offered during four weeks. Both glucose and fructose diets were enriched with simple carbohydrates, accomplished by lowering complex carbohydrate (corn starch) and sucrose (Table 1). Glucose rich diet was administered to evaluate if changes encountered by fructose feeding were due solely to single carbohydrate overfeeding or to the quality of the carbohydrate. Food and water were offered *ad libitum* to mice housed in collective cages (n = 5-6/cage). Average food intake per mouse was measured daily, and body mass weekly throughout four weeks. Energy efficiency was calculated as [(Δ body weight/ ∑ Kcal ingested) x100].

Table 1: Diets composition.

Ingredients (g/Kg)	Diets		
	Control	Glucose	Fructose
Casein	140	140	140
Cornstarch	620.7	220.7	220.7
Sucrose	100	-	-
Fructose	-	-	500
Glucose	-	500	-
Fiber	50	50	50
Soybean oil	40	40	40
Vitamin mix*	10	10	10
Mineral mix*	35	35	35
Choline bitartrate	2.5	2.5	2.5
L-Cystine	1.8	1.8	1.8
Antioxidant#	0.008	0.008	0.008
Sum, g	1 000.00	1 000.00	1 000.00
Kcal/g	3.81	3.81	3.81

*Vitamin and mineral mix composition are based on AIN-93M.
#Tert-butyl hydroquinone.

Glucose, lipid and hepatic parameters

At the day of euthanasia, blood was obtained from awake 6-hour fasted mice by milking the tail after a little incision on its tip, and blood glucose was assessed by a glucometer (One Touch Ultra, Johnson & Johnson, SP, Brazil). Mice were then deeply anesthetized with ketamine 100.0 mg/kg (Francotar®, Virbac, Brazil) and xylazine 10.0

mg/kg (Virbaxyl® 2%, Virbac, Brazil) ip, and the heart was exposed for blood collection from the right atrium. Blood was allowed to clot, centrifuged (1,500 x g), and the serum was stored at -80°C for total cholesterol, HDL and triglyceride (TG) colorimetric assay (cat# K083, K071, and K117 respectively, Bioclin, Quibasa, Belo Horizonte, Minas Gerais, BR), and insulin Elisa assay (cat#EZRMI-13K, Merck Millipore, Billerica, MA, EUA) according to manufacturer's instructions. Insulin resistance was evaluated by the homeostatic model assessment, where HOMA-IR = [insulin (μU/mL) x glucose (mmol/L)]/22.5 [12], and hepatic TG content was determined as described elsewhere [13]. TyG index, the product of fasting plasma glucose (FPG) and TG was used to further assess insulin resistance and liver steatosis [6,7]. It was calculated as ln[FPG (mg/dL) x TG (mg/dL)/2] [7].

Fat weight and distribution

Interscapular brown fat and white visceral (perigonadal and retroperitoneal) and subcutaneous (inguinal) fat pads were carefully dissected from both sides of the animal, weighed and then immersed in 4% phosphate-buffered formalin pH 7.2 for 48 h. Tissue samples followed routine histological processing, were embedded in paraplast, sectioned at 3 μm thick, and stained with hematoxylin and eosin. To evaluate fat distribution, WAT was considered as ∑ (perigonadal (mg) + retroperitoneal (mg) + inguinal (mg)]), visceral WAT as ∑ (perigonadal (mg) + retroperitoneal (mg)]) and the inguinal depot as subcutaneous WAT.

Adipocyte morphometry

Digital photomicrographs were obtained from histological sections using a Leica DMRBE microscope (Wetzlar, German) coupled to a video camera Kappa (Gleichen, German). Morphometry was performed in the Image-Pro® Plus software v. 5.0 (Media Cybernetics, Silver Spring, MD, USA). For the interscapular BAT, eight nonconsecutive images were acquired to assess brown adipocyte diameter, lipid droplet (LD) diameter, and the percentage of tissue area occupied by LD. For lipid area, a selection tool was used to indicate the pixels that represented the LDs, and the pixels selected were segmented in a new digital image in black and white, where the white color represented LDs, and the black one represented the remaining tissue. The white color area was quantified by the image histogram tool. In WAT, adipocyte size was assessed by measuring their smallest and largest diameter, as previously described [14]. Six animals were used per group, four nonconsecutive images per animal, and ten adipocytes were randomly measured per image, summing 40 adipocytes per mice.

Statistics

Data are expressed as mean ± SEM. Data were tested for normality and homoscedasticity of variances. Comparison among the three groups was made by ANOVA one-way followed by a post-hoc test of Tukey. The paired *t*-test compared data obtained on day 0 against day 30 within the same group. A *P*-value of 0.05 was considered statistically significant. All analysis was performed by the GraphPad® Prism software v. 6.0, La Jolla, CA, USA.

Results

Early signs of fructose overfeeding: body fat redistribution without body weight gain

Despite glucose and fructose diets were isoenergetic compared to the control diet, table 2 shows that the cumulative food intake (FI) of fructose group was 10% lower than the control group ($P < 0.02$). Additionally, energy efficiency reduced by 63% and 87%, respectively, in the glucose and fructose groups ($P < 0.0001$). Neither glucose nor fructose feeding changed body weight. Surprisingly, the experimental diets decreased BW gain, since Δ BW was 63% and 88% lower in glucose and fructose groups, respectively, compared to the control group ($P < 0.0001$). All white fat depots studied did not change weight and presented adipocyte hypertrophy as well after glucose or fructose feeding. However, fructose modulated body fat distribution, since the ratio between visceral and subcutaneous WAT increased, compared to the control group (+15%, $P = 0.016$).

Short-term simple carbohydrate feeding has limited impact on glucose, lipid and hepatic metabolism

Thirty days of glucose or fructose feeding did not change glucose and lipid metabolism, as shown in table 3. The glucose group had a decrease of 41% in blood glucose ($P = 0.0002$), that was followed by an increase in serum (+80%, $P < 0.0001$) and liver (+79%, $P = 0.0002$) TG, whereas fructose solely changed total cholesterol (+32%, $P = 0.002$). Insulin resistance was not detected after four weeks of glucose or fructose feeding, as shown by HOMA-IR and TyG index. Although fructose feeding increased liver weight, the TyG index did not indicate hepatic steatosis (Table 3), and we also did not find histopathological changes compatible with steatosis (data not shown).

Table 2: Energy intake, body weight, and adiposity.

Parameters	Groups		
	Control	Glucose	Fructose
Ingestion			
Σ FI, g	115.86±1.92	116±1.85	103.79±2.7#
EE, mg/Kcal	5.97±0.88	2.20±0.68*	0.76±0.36#
Body weight			
Initial, g	30.36±0.46	30.99±0.61	31.36±0.46
Final, g	32.99±0.29	31.96±0.52	31.68±0.54
Δ, g	2.63±0.39	0.97±0.31*	0.32±0.15#
WAT			
Genital, mg/g of BW	12.65±0.82	12.05±0.78	14.04±0.46
Retroperitoneal, mg/g of BW	3.78±0.30	3.29±0.22	4.10±0.32
Inguinal, mg/g of BW	11.51±0.68	10.88±0.83	11.38±0.66
Visc: Subc ratio	1.44±0.06	1.43±0.06	1.65±0.05*#
Adipocyte diameter			
Genital, μm	59.43±1.69	56.43±1.84	57.65±0.96
Retroperitoneal, μm	54.03±0.73	52.99±3.02	54.59±1.50
Inguinal, μm	42.27±2.51	37.79±2.41	35.68±1.98

Data are expressed as mean ± SEM. When signaled, $P < 0.05$, [*] vs. Control, [#] vs. Glucose (one-way ANOVA and Tukey's multiple comparisons). Abbreviations: Σ FI, the sum of food intake from day 0 to the 30th day; EE, energy efficiency; WAT, white adipose tissue; BW, body weight; Visc, visceral fat; Sub, subcutaneous fat. Energy efficiency was calculated based on Δ body weight and Σ energy intake.

Table 3: Glucose, lipid and hepatic metabolism.

Parameters	Groups		
	Control	Glucose	Fructose
Glucose metabolism			
Glucose (day 0), mg/dL	128±7.11	116.9±7.04	107±4.31
Glucose (day 30th), mg/dL	120.6±8.56	68.6±5.45*&	111±5.67#
Insulin, ng/mL	0.32±0.01	0.29±0.02	0.38±0.01
HOMA-IR	2.66±0.24	2.44±0.25	2.93±0.18
Lipid metabolism			
Total Cholesterol, mg/dL	92.64±4.34	95.61±3.11	122.1±5.81*#
HDL, md/dL	47.16±2.04	49.11±3.13	41.2±4.51
Triglyceride, mg/dL	71.49±4.18	128.7±15.51*	65.92±3.62#
Hepatic metabolism			
Liver, g/g BW	0.042±0.0006	0.041±0.0007	0.044±0.0006*
Triglyceride, mg/dL/mg	2.76±0.34	4.95±0.54*	2.78±0.20#
TyG index	8.37±0.13	8.31±0.07	8.11±0.09

Data are expressed as mean ± SEM. When signaled, $P < 0.05$, [*] vs. Control, [#] vs. Glucose (one-way ANOVA and Tukey's multiple comparisons), and [&] vs. day 0 (paired *t*-test). Abbreviations: BW, body weight; TyG, the product of glucose and triglyceride.

Early changes in BAT morphology due to short-term fructose feeding

Figure 1 shows that BAT weight remained unchanged after four weeks of glucose or fructose feeding, as well as the ratio between BAT and WAT, despite a subtle decrease of this last parameter in the fructose group. When the amount of visceral WAT is compared to BAT mass, it decreased by 18% in the fructose group compared to the control group (P = 0.03).

Figure 2 shows that thirty days of glucose or fructose feeding did not change brown adipocyte size (Figure 2A), but intracellular lipid deposition is noticed after glucose and fructose feeding (Figure 2B). The percentage of BAT area occupied by LDs increased by 19% (P = 0.01) in the glucose group and 17% (P = 0.02) in the fructose group. In the fructose group, this increase can be attributed to LD hypertrophy, since average LD diameter increased 17% (P = 0.006) compared to the control group (Figure 2C). BAT photomicrographs in figure 2D-I show that lipid droplets have a uniform size and are evenly distributed in control mice (D,G). Although the glucose group did not present lipid droplet hypertrophy (Figure 2B), some large droplets are noticed in figure 2H. Finally fructose feeding lead to lipid droplet hypertrophy as seen in figures 2E-F.

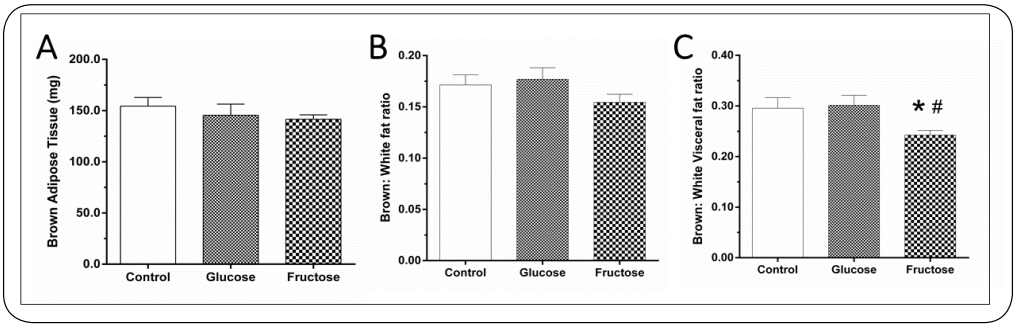

Figure 1: Interscapular brown fat. Glucose or fructose feeding neither change brown adipose tissue (BAT) mass (A) nor the ratio between BAT and white adipose tissue (WAT) mass (B). However, fructose decreased the ratio between BAT and visceral WAT (C). N = 6 mice/group, mean ± SEM, One-way analysis of variance, and the post-hoc test of Tukey, P < 0.05, [*] vs. Control, [#] vs. Glucose.

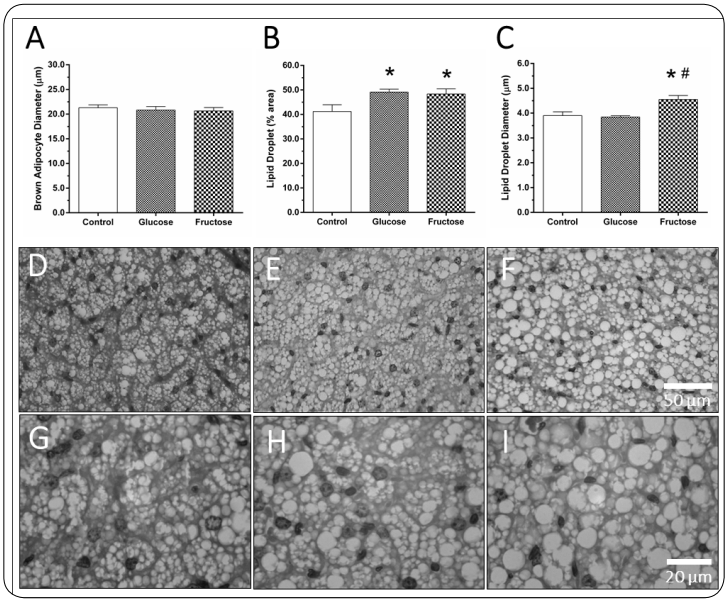

Figure 2: Diet-induced morphological changes in interscapular brown adipose tissue (BAT) of male C57BL/6 mice. Brown adipocyte size did not change by glucose or fructose feeding (A), but both diets increased the percentage of tissue area occupied by cytoplasmic lipid droplets (B). In fructose-feed mice, lipid droplet diameter increased (C). D-I show photomicrographs in lower (D,E,F) and higher (G,H,I) magnification illustrating BAT remodeling (hematoxylin and eosin stain). Lipid droplets have a uniform size distribution in control mice (D,G). Although glucose feeding did not change the average lipid droplet size, some large droplets are noticed (E,H). On the other hand, lipid droplet hypertrophy is visible in E and F due to fructose feeding. N = 6 mice/group, mean ± SEM, One-way analysis of variance, and the post-hoc test of Tukey, P < 0.05, [*] vs. Control, [#] vs. Glucose.

Discussion

We demonstrated in male C57BL/6 mice that short-term fructose feeding did not promote weight gain and adiposity, but WAT fat was redistributed to visceral depots. Dysfunction of glucose, lipid, and hepatic metabolism was not noticed, except for increased serum total cholesterol and liver weight. The most prominent finding is that fructose feeding changed the ratio between BAT and visceral WAT, where the second predominated, and BAT suffered a significant early morphological remodeling due to increased lipid deposition in enlarged intracellular LD. Of note, a limitation of the present study is that the experiment was not performed under thermoneutrality conditions.

In the present study, fructose reduced cumulative food intake, and it likely justifies the absence of BW gain. Tillman and others [15] showed that even when cumulative food intake increased, BW gain is not seen after 14 weeks of 60% fructose feeding. The same group showed that the metabolic rate increased at the second and ninth weeks of fructose feeding, but at the 14^{th} week, the metabolic rate was similar to the control group. We showed through the energy efficiency that the fructose group gained less weight per energy consumed, compared to control group, and based on Tillman's work we suppose that fructose might have also increased metabolic rate thus maintaining the BW unchanged.

After short-term fructose feeding (four weeks), C57BL/6 mice did not present either increased adiposity or adipocyte hypertrophy, although early signs of fat redistribution were found toward increased visceral WAT. Long-term fructose feeding is supposed to promote WAT gain, and Montgomery and others [16] showed an increased mass of visceral (perigonadal and retroperitoneal) and subcutaneous (inguinal) WAT depots after eight weeks of fructose feeding. Visceral and subcutaneous WAT have distinct functions, and traditionally, visceral WAT is associated with lipid and glucose metabolic disturbances and cardiovascular risk [17]. For instance, visceral obesity contributes to insulin resistance and coronary artery disease development in nonobese individuals [18]. However, Moreno-Indias and others [19] showed in normal-weight subjects that macrophage-associated genes are upregulated in subcutaneous but not visceral WAT. It suggests that macrophages within subcutaneous fat may contribute to the glucose and lipid metabolic disturbances seen in MONW individuals.

There is substantial evidence that fructose overfeeding promotes the development of glucose intolerance, insulin resistance, and hepatic steatosis as reviewed elsewhere [20]. In the present study, hypercholesterolemia was the single metabolic change found after short-term fructose feeding. We believe that the time required for fructose to induce metabolic abnormalities, such as insulin resistance, needs to be longer than four weeks. Montgomery and others [16] showed in mice that long-term fructose feeding (eight weeks) did not change fasting glucose and insulin, but it promoted glucose intolerance and decreased plasma TG and non-esterified fatty acids (NEFA). Additionally, Tillman and others [15] showed that glucose, triglycerides, and NEFA did not change after 14 weeks of fructose feeding in C57BL/6 mice. Although fructose stimulates gluconeogenesis, it seems to produce only mild changes in blood glucose [21]. Fructose would not increase blood insulin levels acutely because it does not induce pancreatic beta cell secretion of insulin as does glucose, but chronic exposure to fructose might lead to hyperinsulinemia [22].

Insulin resistance encompasses impairment of fatty acid oxidation and utilization [23], and the hepatic TG content is a strong determinant of hepatic insulin resistance [24]. In the present study, short-term fructose intake increased liver weight despite no signs of hepatic steatosis, as suggested by the TG content, TyG index, and histopathological analysis (data not shown). Long-term studies have shown that fructose feeding promotes hepatic lipid accumulation of TG and DAG, but not ceramides [16]. The phenomenon is due to the upregulation of lipogenic pathways through the elevation of the protein content of both acetyl-CoA carboxylase isoforms (ACC1 and ACC2), fatty acid synthase (FAS) and stearoyl-CoA desaturase (SCD1) [16].

Short-term fructose feeding did not change BAT mass, a result found by us and others [16]. We have previously shown in female C57BL/6 mice that BAT mass gain is a late event in BAT dysfunction during aging [13]. We also showed in the present study that the average LD size was increased by fructose, but cell size was not affected. We suppose that long-term fructose feeding would result in brown adipocyte hypertrophy, as well as white adipocyte hypertrophy. In aged male C57BL/6 mice, brown adipocyte hypertrophy is associated with BAT morphological adverse remodeling, diminished thermogenesis, and glucose intolerance [25]. Lipid droplet hypertrophy is due to increased TG storage and, in humans, the TG content of thermogenic supraclavicular fat deposits

may be an independent marker of whole-body insulin sensitivity, independent of BAT metabolic activation [26]. In summary, these data show early modifications of BAT adipocyte architecture by fructose, that will likely lead to metabolic dysfunction in the future, but additional studies are necessary to confirm this hypothesis.

Lipid droplet size regulation and lipid storage capacity are essential to maintain normal biological functions, and its dysregulation results in the development of metabolic diseases such as obesity, diabetes, fatty liver, and cardiovascular diseases. The nutritional response, hormones, and environmental factors may also contribute to increased LD size and lipid storage [27]. Lipid droplet growth occurs by the fusion of pre-existing LDs, lipid biosynthesis in situ or by lipid transfer from adjacent organelles, including endoplasmic reticulum. Although white and brown adipocytes accumulate large amounts of fat, LDs differ in size, number, and protein content [28]. Brown adipocytes are filled with many relatively small sized LDs (multilocular) that are closely associated with mitochondria. This arrangement facilitates the accessibility of lipases to LD surface for the fast release of fatty acids. Lipid droplet biosynthesis and expansion are driven by complex and integrated mechanisms involving interactions with other organelles and enzymes for the expansion of the lipid core and the modulation of phospholipid monolayer composition [29].

Finally, in C57BL/6 mice, it seems that fructose *ad libitum* in the drinking water can elicit an obesogenic response, but not when it is offered in rodent chow. Thus, further studies are necessary to elucidate the mechanisms underlying fructose-induced obesity, inflammation and glucose, lipid, and hepatic metabolic disturbances in rodents based the route of fructose administration.

Conclusion

Short-term fructose feeding redistributes fat among WAT depots, alters the ratio between BAT and visceral WAT, and promotes BAT adverse remodeling, characterized by enlarged intracellular lipid droplets, in young male C57BL/6 mice. The novelty of the present work is that BAT displays early morphological signs of tissue dysfunction that likely precedes the onset of glucose and lipid metabolic disturbances.

Declarations

- The local Ethics Committee approved the handling and experimental protocols to Care and Use of Laboratory Animals (CEUA#647/15).
- All authors read and consent publication.
- There are no conflicts of interest to declare.
- The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.
- Fernandes-Santos C conceived and designed the experiments; Machado TQ and Goncalves LF performed the experiments; Fernandes-Santos C and Pereira-Silva DC analyzed and interpreted the data, and wrote the paper.
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