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Oral Fat Tolerance Test in Male and Female C57BL/6 Mice

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[Abstract] Postprandial hyperlipidemia is an important risk factor for cardiovascular diseases, and it can be evaluated by an oral fat tolerance test (OFTT). There is no consensus on how to perform the OFTT in rodents, especially in the C57BL/6 mice strain. Furthermore, there is no consensus in the literature regarding several variables involved in the test (*e.g.*, dietary lipid source and fasting duration), and consequently, there is no standardization for the OFTT protocol. Therefore, we aimed to demonstrate a protocol for OFTT in male and female C57BL/6 mice. Briefly, mice underwent a 2 h-fasting period followed by blood collection from the tail and orogastric gavage of 200 µl soybean oil. Blood was collected again at 1, 2, and 3 h after gavage, and centrifuged for triglyceride quantitation through a colorimetric enzymatic assay. Triglyceridemia was evaluated throughout the test, and the area under the curve was calculated. The present protocol contributes to standardizing the OFTT protocol in C57BL/6 mice and enables future research regarding the influence of sex and cardiometabolic diseases on postprandial lipemia.

Keywords: Fatty acids, Lipid metabolism, Oral fat tolerance test, Postprandial triglyceridemia, Postprandial lipemia, Triglycerides.

[Background] The amount of triglycerides (TG) produced in lipid metabolism has an important correlation with the onset and development of cardiometabolic diseases. Postprandial lipemia is an important cardiovascular risk biomarker since, among the usual blood lipid fractions analyzed, TG is the first to change after food intake (Nordestgaard, 2016). Recently, international associations recommended that fasting is no longer required for blood lipid analysis. It is justified firstly by the fact that individuals remain most of their time in a postprandial state, and secondly because there is no significant variation in TG between fed or fasted individuals (Nordestgaard *et al.*, 2016). The oral fat tolerance test (OFTT) is a reliable method for assessing postprandial lipemia because it represents the body's capacity to normalize hyperlipidemia after lipid intake, allowing a more profound analysis of lipid metabolism. The OFTT consists of giving a particular food and monitoring its metabolic response in the body (Langsted and Nordestgaard, 2011).

Animal models are essential for studying metabolism and cardiometabolic diseases; however, there is currently no consensus regarding how to perform the OFTT in rodents, and as a consequence, there is a wide range of variation among published OFTT protocols. A recent study demonstrated that OFTT results are influenced by mouse strain, dietary lipid source, fasting duration, and sex (Ochiai, 2020).

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Although Ochiai *et al.* indicated that other strains may have a better response in OFTT (Ochiai, 2020), C57BL/6 mice continue to be the most studied strain for cardiometabolic diseases (Fazio and Linton, 2001); therefore, it has become crucial to establish a protocol to assess postprandial lipemia in these animals.

The vast majority of OFTT studies performed in C57BL/6 mice use olive oil (Kimura *et al*., 2011 and 2013; Toyoda-Ono *et al*., 2007; Hiel *et al*., 2018; Sairyo *et al*., 2018); although, there are some reports with coconut (enriched with oleic acid) (Hernandez Vallejo *et al*., 2009), safflower (Yamazaki *et al*., 2012), and corn (King *et al*., 2010) oils. Ochiai *et al.* demonstrated that olive and soybean oils present similar OFTT results in ddY mice (Ochiai, 2020). It is also important to consider the amount of fat administered, since the dose reported in the literature ranges from 5 ml/kg to 17 ml/kg (Toyoda-Ono *et al*., 2007; King *et al*., 2010; Sairyo *et al*., 2018) and the volume ranges from 150 µl to 400 µl (Hernandez Vallejo *et al*., 2009; Kimura *et al*., 2011 and 2013; Yamazaki *et al*., 2012). In the face of this evidence, we propose an OFTT meal protocol of 200 µl soybean oil, which corresponds to approximately 7 ml/kg. We did not correct the oil volume by body weight because we noticed that body mass fluctuations within the range expected for this mouse strain did not significantly alter the volume of oil administered, and it is also difficult to measure precisely in the syringe.

The fasting duration can range from 4 h to 24 h (Toyoda-Ono *et al*., 2007; Hernandez Vallejo *et al*., 2009; King *et al*., 2010; Kimura *et al*., 2011 and 2013; Yamazaki *et al*., 2012; Sairyo *et al*., 2018). Considering that fasting is a stressful event for rodents (Choi *et al*., 2005) and current guidelines for humans suggest that fasting is no longer required for TG analysis (Nordestgaard *et al*., 2016), we propose a short fasting protocol of 2 h. Lastly, sex should also be considered, since most studies are conducted in male mice (Toyoda-Ono *et al*., 2007; Hernandez Vallejo *et al*., 2009; King *et al*., 2010; Kimura *et al*., 2011 and 2013; Yamazaki *et al*., 2012; Hiel *et al*., 2018; Sairyo *et al*., 2018). Ochiai *et al.* showed that females are less prone to lipid-induced hypertriglyceridemia; however, these results were reported only in ddY mice (Ochiai, 2020), thus, we chose to propose a protocol for male and female mice.

Another two important factors to consider are the frequency and timing of blood sampling and mouse metabolic background. Concerning the former, the TG peak would appear between 2 h and 3 h after lipid administration (Yamazaki *et al*., 2012; Sairyo *et al*., 2018; Ochiai, 2020). Regarding the latter, some studies conducted the OFTT in high fat-fed mice, while others were performed in healthy mice. According to Ochiai *et al*., high-fat and high-sucrose feeding for one week is sufficient to promote lipid-induced hypertriglyceridemia during the OFTT (Ochiai, 2020).

Based on these data, we aimed to demonstrate a protocol for OFTT in male and female C57BL/6 mice using 200 µl soybean oil after a 2-h fasting period.

Materials and Reagents

1. 96-well plates for 0.3 ml volume with flat-bottomed wells (Olen, catalog number: K30-5096P)
2. 1-ml slip tip disposable tuberculin syringe (BD, catalog number: 309659)

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3. Disposable scalpel blade (Solidor, model #10, Brazil)
4. 0.5-ml microtube (Axygen™, catalog number: 14-222-292)
5. Pipette tips (P-10, P-100, P-200) (Labmate, Saint Albans, UK)
6. Mouse restrainer (Insight, catalog number: EB286CO)
7. Paper towel
8. Sterile gauze
9. Adult C57BL/6 mice (≥ 3 months old)
10. Soybean oil (Liza, Curitiba, Brazil)
11. Ultrapure water (MilliQ® Direct Water Purification System, Merck, Darmstadt, Germany)
12. Ice
13. Triglyceride reagent kit (catalog #26B, Interteck, São Paulo, Brasil, GPO/PAP)

Equipment

1. Ventilated cages (Scienlabor, catalog number: SLB50.56)
2. Reusable curved animal feeding needle (Cadence Science™, model: 01-290-9A)
3. Microtube rack (Axygen™, model: 14-222-391)
4. Refrigerated centrifuge (Cientec, model: CT-1500)
5. Vortex mixer (IKA, model: K45-2820)
6. Water bath (Novatecnica, model: NT-245)
7. Epoch UV-visible light spectrophotometer (BioTek® Instruments, Vermont, USA)
8. Refrigerator (Consul, CRD37EBANA, São Paulo, Brazil)

Software

1. Gen5™ Microplate Data Collection & Analysis Software (BioTek® Instruments, Vermont, USA)
2. Microsoft Excel (Microsoft, Redmond, Washington, EUA)
3. GraphPad Prism v.8.0 (GraphPad Software, San Diego, California, USA)

Procedure

A. Animal housing conditions

Prior to the procedures, animals were maintained in ventilated cages (Scienlabor, Ribeirão Preto, São Paulo, Brazil) under controlled conditions of 21±1°C, 60±10% humidity, 15 min/h exhaustion, and 12 h dark/light cycle (artificial lights, 7am-7pm).

B. Oral fat tolerance test (OFTT) (Figure 1)

1. Fast the mice for 2 h.
2. Cut the tip (1 mm) off the tail with a scalpel blade.

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3. Milk the tail and collect the blood drops using a pipette (P10).
4. Dispense the blood in a 0.5-ml microtube.
5. Repeat the process to obtain about 20 µl blood (~4 blood drops).
6. Perform orogastric gavage of 200 µl soybean oil in each animal (video showing the procedure: https://youtu.be/oYCmKIhveFY).
7. Take 20 µl blood from the tail (~4 blood drops) using a pipette (P10) at 1 h, 2 h, and 3 h after orogastric gavage.
   Note: If the original tail cut is healed or has a blood clot, remove the blood clot or cut the tip (1 mm) off the tail with a scalpel blade.
8. Store the blood in a 0.5-ml microtube at room temperature for 20 min and then on ice until centrifugation.
9. Centrifuge blood samples (800 × *g* for 10 min).
10. Reserve the serum in a new 0.5-ml microtube.
    Note: Keep it on ice if the assay is performed on the same day or store it in the refrigerator at 4°C (for up to one month) until the biochemical assay.

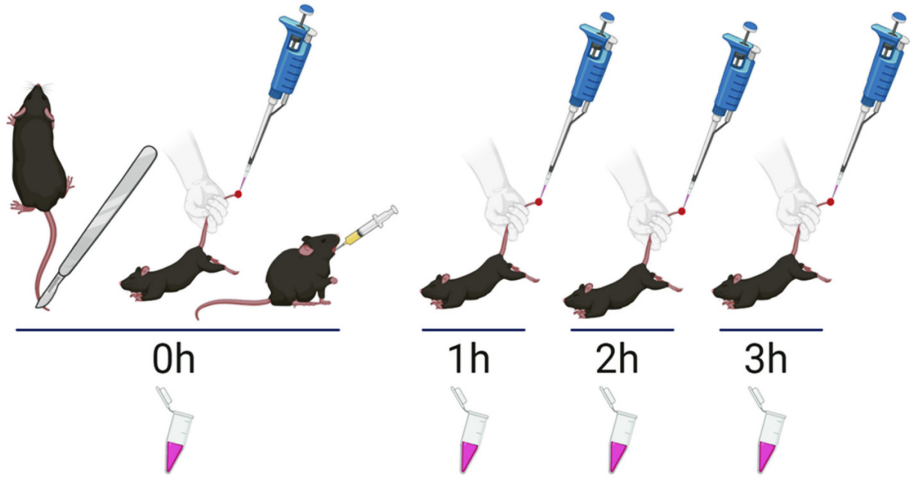

Figure 1. Oral fat tolerance test (OFTT). Image created in BioRender.com.

C. Biochemical analysis (Tables 1 and 2)
1. Remove the serum from the refrigerator and keep it on ice or in a thermal bag throughout the assay.
2. Reserve the TG reagent kit, which consists of a control sample (100 mg/dl) and a working reagent, and allow them to equilibrate to room temperature (for reagent composition, see Table 1).
3. Prepare controls #1, #2, and #3 by diluting the control sample provided in the kit, which will be used later to obtain the standard curve equation (see Table 2).
   a. Use three controls: the one provided in the kit (control #1) and two further concentrations

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(controls #2 and #3).

b. Dilute the control sample with ultrapure water at concentrations of 1:2 and 1:4.

4. Organize sample disposition in the 96-well plate.
   a. Include blank and control samples in triplicate and test samples in duplicate.
5. Pipette 200 µl working reagent in all wells.
6. Pipette 2 µl ultrapure water in blank wells (triplicate).
7. Pipette 2 µl either control (triplicate) or samples of unknown concentration in the corresponding well (duplicates).
   Note: Use a vortex to homogenize the sample before pipetting.
8. Incubate the microplate in a water bath at 37°C for 10 min.
9. Perform microplate reading in the light spectrophotometer at a wavelength of 505 nm and area scan 5 × 5.

Table 1. TG reagent kit composition*

Kit reagent	Composition
Control sample	• 2.26 mmol/l Glycerol • 0.1 g/dl Sodium azide
Working reagent	• 50 mmol/L PIPES buffer pH 7.0 • ≥ 4,000 U/L Lipoprotein lipase • 2 mmol/l ATP • 0.3 mmol/l 4-Aminoantipyrine • ≥ 1,000 U/L Glycerol kinase • ≥ 3,000 U/L Glycerol phosphate oxidase • ≥ 2,010 U/L Peroxidase • 2.70 mmol/L p-Chlorophenol • 0.95 g/dl Sodium azide

* According to the manufacturer.

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Table 2. Assay reaction

	Working reagent	Control sample	Ultrapure water	Samples of unknown concentration	triglyceride
Blank	200.0 µl	-	2.0 µl	-	0.0 mg/dl
Control #1	200.0 µl	2.0 µl	-	-	100.0 mg/dl
Control #2	200.0 µl	1.0 µl	1.0 µl	-	50.0 mg/dl
Control #3	200.0 µl	0.5 µl	1.5 µl	-	25.0 mg/dl
Sample	200.0 µl	-	-	2.0 µl	unknown

Absorbance of controls #1, #2, and #3 will be used to calculate the standard curve equation.

Data analysis

A. Calculations

1. Average absorbance: The 5 × 5 reading will generate 21 readings for each well. First, remove the readings that correspond to the well border (columns #1 and #5, lines #1 and #5) and then calculate the average absorbance for each well. The reason is that the TG reagent sometimes produces bubbles that are trapped at the well edge, increasing the average well absorbance since the equipment reading is performed along the vertical axis. Subsequently, calculate the average absorbance of triplicates/duplicates of each control and test samples.
2. To calculate triglyceride concentration: First, calculate the standard curve equation from the control samples using linear regression. In GraphPad Prism, choose the ‘XY table’ and plot the control sample concentration (X) against absorbance (Y). Run the linear regression analysis and find the curve equation (Y = aX + b, where the statistical software provides ‘a’ and ‘b’). For an example of the standard curve, please see Figure 2. Subsequently, in Microsoft Excel, calculate the unknown sample TG concentration (mg/dl) by substituting ‘Y’ in the equation with sample absorbance.

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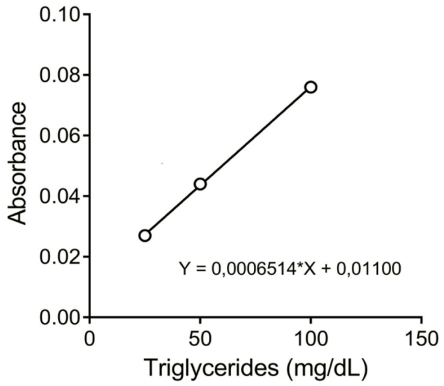

Figure 2. Example of the standard curve. In the graph, the control sample concentration (X) and absorbance (Y) are plotted. Control sample concentrations consist of the one provided with the kit (100 mg/dL) and two other dilutions (1:2 and 1:4). The absorbances were obtained by reading in the light spectrophotometer at a wavelength of 505 nm. Linear regression analysis was performed to find the curve equation (Y = aX + b). Goodness of the fit of linear regression: R^2 = 0.9998; Sy·x = 0.0005345.

3. OFTT curve: In GraphPad Prism, use the 'XY table' to plot the TG time–response curve. Time in hours (X) is plotted against TG concentration in mg/dl (Y). Use the mean and standard deviation of the TG concentration of the control and experimental groups.
4. OFTT net incremental area under the curve (AUC): Includes all the incremental area below the curve, including the area below the fasting concentration. It is calculated using the trapezoidal rule for positive and negative increments (Gannon *et al*., 1989). In GraphPad Prism, create a 'XY table,' add the time (0, 1, 2, and 3 h) in the 'X' column and TG in the 'Y' column (one animal per column, use one table to add data from one experimental group), and run the 'analyze > area under the curve.' Subsequently, copy the data 'total area' from the results sheet and paste it to a new 'column table,' where each column is an experimental group, to run the appropriate statistical test for group comparisons.
5. OFTT incremental area under the curve (iAUC): The iAUC includes all areas below the curve and above the fasting concentration, with any area beneath fasting being ignored (Wolever and Jenkins, 1986). In GraphPad Prism, create an 'XY table,' plot time (0, 1, 2, and 3 h) in the 'X' column and TG in the 'Y' column (one animal per table). Run the 'analyze > area under the curve,' and in the dialogue box, type the TG concentration for that mouse at time 0 h in the 'Baseline Y = ' box. Subsequently, copy the data 'total peak area' from the results sheet and paste it to a 'column table,' where each column is an experimental group, for group comparisons.

B. Data obtained

Herein, we show our results for the OFTT (Figure 3), body mass, and glucose (Figure 4). We used saline as a negative control for the OFTT, but this comparison is not required when using the test to evaluate postprandial lipemia. Moreover, we also reported the changes in body mass and glycemia

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during the OFTT so readers are aware that while there is a reduction in body mass, blood glucose is not altered during the test. The glucose AUC was calculated by applying the same steps described for the OFTT AUC.

For OFTT, we compared time intervals in the same group and the groups at each time point. We also evaluated the TG AUC and iAUC and differences (ΔTG) at 1 h vs. 0 h and 2 h vs 0 h. In males, the TG of the soybean group at 3 h was reduced by 19% in comparison with that at 2 h. In females, the TG at 2 h was increased by 29% with soybean oil as compared with saline. Soybean oil elevated the TG AUC (+24%) and iAUC (+911%) at Δ1 h vs. 0 h and Δ2 h vs. 0 h.

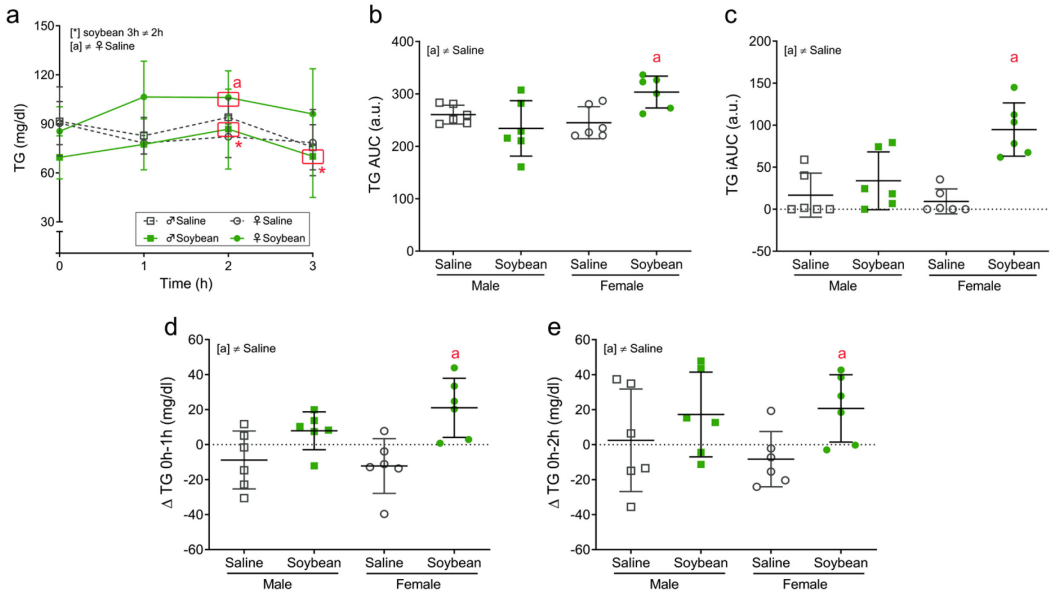

Figure 3. Oral fat tolerance test (OFTT). (a) Triglyceride (TG) response curve during the OFTT in mg/dl. In (b) and (c), the area under the curve (AUC) and the incremental AUC (iAUC) were calculated for group comparisons. In (d) and (e), the TG response from 0 h until the first (d) and second (e) hours are presented.

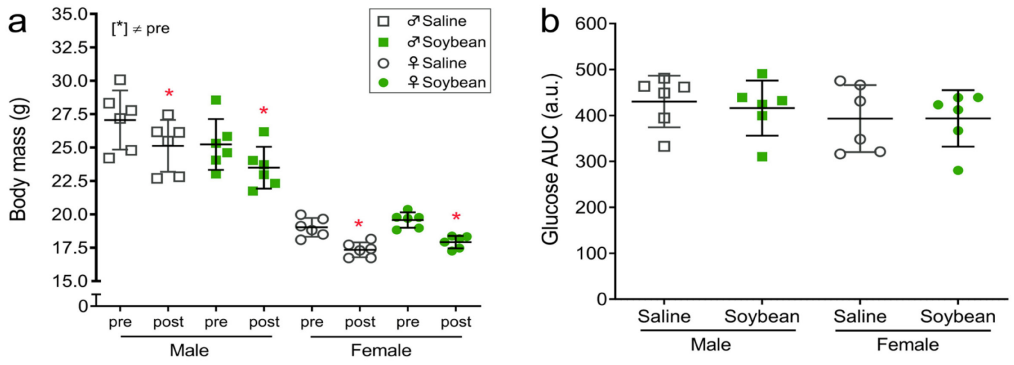

Figure 4. Body mass and glucose. (a) Body mass of male and female C57BL/6 mice assessed

before a 2h-fasting and at the end of the OFTT. They lost 7.0 ± 1.6% (male) and 8.6 ± 0.9% (female) of their body mass during this time range. (b) The area under the curve (AUC) of glucose was not changed by saline or soybean oil during the OFTT.

Notes

Caution has to be taken during blood collection because successive tail milking may lead to blood hemolysis. It is also important to control the room temperature so that the body temperature of the mice is stable, otherwise the amount of blood collected is insufficient for biochemical assays. Mice of different sexes, ages, and inbred backgrounds can show different responses to OFTT. The protocol was performed in control female and male mice; thus it is important to establish the OFTT response in diet-induced obesity models. Lastly, in theory, other lipids could be assessed by the OFTT. Nevertheless, studies show that TG present relevant acute variations in the blood after food intake and thus, studying postprandial TG is important for predicting the presence of risk factors for cardiovascular diseases (Lopez-Miranda *et al.*, 2007; Ochiai, 2020). Based on these data, researchers should be aware of analyzing other lipids in the OFTT before including the test in their procedures.

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Competing interests

The authors have no competing interests to declare.

Ethics

The experiments were approved by the local Ethics Committee (Fluminense Federal University, protocol number 920/2017).

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