



Functional Tests for Assessing Human Beta-Cell Function and Insulin Sensitivity

Marcelo Miranda de Oliveira Lima and Bruno Geloneze

Abstract

Glucose homeostasis requires functional tests for assessing pancreatic beta-cell function and insulin sensitivity. The hyperinsulinemic-euglycemic clamp technique is the “gold-standard” method for insulin sensitivity, used to validate other methods. The full comprehension of beta-cell function complexity requires different intravenous or oral tests and the assessment of insulin sensitivity, which can be evaluated simultaneously with insulin secretion by most methods. The selection of the methods of assessing glucose homeostasis in the research settings depends on the aspects of beta-cell function and insulin sensitivity relevant to the proposed hypothesis.

Key words Insulin sensitivity, Insulin secretion, Glucose clamp technique, Glucose tolerance test, Insulin-secreting cells, Insulin, C-peptide

1 Introduction

The study of glucose homeostasis requires functional tests for assessing pancreatic beta-cell function (insulin secretion in response to a given stimulus) and insulin action in tissues (insulin sensitivity). Insulin sensitivity is frequently evaluated independently of insulin secretion, as the former relates to, and is a risk factor for metabolic conditions regardless of glucose tolerance, such as adiposopathy, hypertension, dyslipidemia, cardiovascular disease, systemic inflammation, and polycystic ovarian syndrome, among others [1]. In turn, the beta-cell function has a central role in glucose tolerance and the evaluation of its disorders, particularly diabetes mellitus, requires the assessment of insulin secretion primarily [2]. However, the beta-cell function cannot be correctly interpreted without evaluating the prevailing insulin sensitivity because the maintenance of glucose homeostasis comprises the adaptation of the former to the latter [2, 3].

The hyperinsulinemic-euglycemic clamp is considered the “gold-standard” method of assessing insulin sensitivity and is used for validation of other methods [1]. Most assessment methods of beta-cell function also permit the estimation of insulin sensitivity in a single experiment [2]. A representative method is a hyperglycemic clamp, in which the insulin sensitivity index is similar to that of the hyperinsulinemic-euglycemic clamp [2, 4]. Other methods estimate insulin sensitivity using empirical formulas and computer-assisted calculations that are freely available and easy to use, or mathematical models that are more reliable but require specific software and expertise [5, 6].

Indexes of beta-cell function or insulin sensitivity must be calculated from independent time periods during the experiment; otherwise, the correlation between these indexes would be biased by collinearity resulting from coincident values of glucose and insulin or C-peptide used in the calculation of both indexes [2].

None of the assessment methods of beta-cell function may be considered “gold standard” because of the multiple aspects of insulin secretory response that cannot be evaluated in a single test [5]. The complexity of the beta-cell function is illustrated by the many possibilities of stimulation or potentiation agents (glucose, other macronutrients, incretins, glucagon, pharmacologic secretagogues, or mixed stimuli), routes of administration (oral versus intravenous, or combined), or standardization of the stimulus.

Intravenous standardized methods like the intravenous glucose tolerance test (IVGTT) and the hyperglycemic clamp have the advantage of not requiring mathematical adjustment of the insulin secretory response to a variable, dynamic stimulus, as do the oral tests, which may be imprecise. The hyperglycemic clamp is one of the preferred methods because it gathers standardized stimulus during the whole experiment and accurate outcomes, and it does not require complex mathematical models for concomitant assessment of insulin sensitivity [2, 4].

Oral stimulation is considered more physiological because it is the natural route of glucose and other nutrients and because a fundamental component of insulin secretory response is the entero-insular axis. It integrates neural pathways involved in glucose homeostasis and gut hormones that markedly potentiate insulin secretion, the so-called incretin effect, particularly glucose-dependent insulinotropic peptide (GIP) and glucagon-like peptide-1 (GLP-1) [7]. For these reasons, oral tests are particularly useful for studying glucose tolerance disorders, bariatric/metabolic surgery, the development of pharmacological and nutrition therapies, and food science. A limitation is that oral tests cannot distinguish intrinsic beta-cell defects from those of the entero-insular axis. In addition, the secretory stimulus cannot be standardized (mostly the variable glucose levels), requiring more complex methods for normalization of insulin secretion to the stimulus [5].

The beta-cell function can be determined from insulin or C-peptide concentrations. Insulin measurements are frequently employed due to facility, but they do not correspond to the full insulin secretion because near half of it undergoes hepatic extraction before reaching the peripheral circulation, a major component of insulin clearance. Changes in hepatic extraction in diverse metabolic conditions may account for much of the variation in peripheral insulin [8]. Nevertheless, peripheral insulinemia correlates well with pancreatic insulin release, particularly during rapid insulin secretion, such as that seen in the first phase secretion elicited by intravenous glucose load.

To overcome the limitations related to hepatic insulin clearance, C-peptide measurements are used in a mathematical model (C-peptide deconvolution) to reconstruct insulin secretion [9]. C-peptide is cosecreted with insulin in equimolar amounts, undergoes negligible hepatic extraction, and has linear and relatively constant kinetics. The beta-cell function index derived from the deconvolution model is the insulin secretion rate (ISR) (pmol/min). The C-peptide levels approach is preferred, but it requires specific software and expertise.

In response to a gradual increase in plasma glucose concentration, insulin secretion is progressively stimulated, and a linear dose-response relationship is observed between glucose concentration and insulin secretion [10]. Based on this principle, beta-cell sensitivity may be calculated. When plasma glucose concentration is sharply increased and maintained at a suprabasal level, as occurs in the hyperglycemic clamp, insulin secretion shows a biphasic pattern, with an initial acute insulin response (AIR), that corresponds to first phase insulin secretion, followed by a gradually increasing secretion that approaches a nearly constant level after about 60–120 min (second-phase insulin secretion) [4]. The magnitude of both first- and second-phase insulin responses relate to the glycemic increment; the magnitude of the second phase relates to the beta-cell dose response. A biphasic response is also observed with the intravenous glucose tolerance test (IVGTT), in which the first-phase acute insulin response (AIR) is followed by a slower and more blunted secretion rise (second phase) [11]. Both first and second phases can be potentiated by previous prolonged exposure to hyperglycemia (beta-cell potentiation) [12]. The beta cells also respond to various nonglucose stimuli. The amino acid arginine is a potent secretagogue. Its acute intravenous administration produces an intense first phase insulin response, potentiated by hyperglycemia, and is considered the “maximal insulin response” [13].

As beta-cell function adapts to the prevailing insulin sensitivity for maintenance of glucose homeostasis, insulin sensitivity must be taken into account in the interpretation of insulin secretion indexes. The most widely used approach is the so-called disposition index (DI), derived from the IVGTT and the minimal model for insulin

sensitivity. The index of beta-cell function corrected for insulin sensitivity (the disposition index) is the product of the acute insulin response (AIR) and the index of insulin sensitivity, derived from the hyperbolic relationship described between AIR and insulin sensitivity index [11]. However, the application of this adjustment requires some caution. The indices of beta-cell function and insulin sensitivity should be based on independent variables (e.g., different functional tests and/or different intervals of the same test), and whether the relationship between them is exactly a hyperbola should be verified in the studied sample. Nevertheless, correlations of other variables with the disposition index may depend on insulin sensitivity rather than the beta-cell function [2, 6].

2 Intravenous Glucose Tolerance Test (IVGTT)

The intravenous glucose tolerance test (IVGTT) is performed by a single glucose infusion in a rapid bolus to stimulate a sharp pattern of the first-phase insulin secretion, followed by a second phase (Fig. 1). For assessment of the first phase, a 10-min IVGTT is sufficient. However, the IVGTT is often used to evaluate insulin sensitivity with the minimal model and the second-phase secretion [11].

The first phase of insulin secretion is assessed in the initial 8–10 min, with frequent samples (at 1–2 min intervals), beginning 2–3 min after time zero. The most widely used first-phase index is

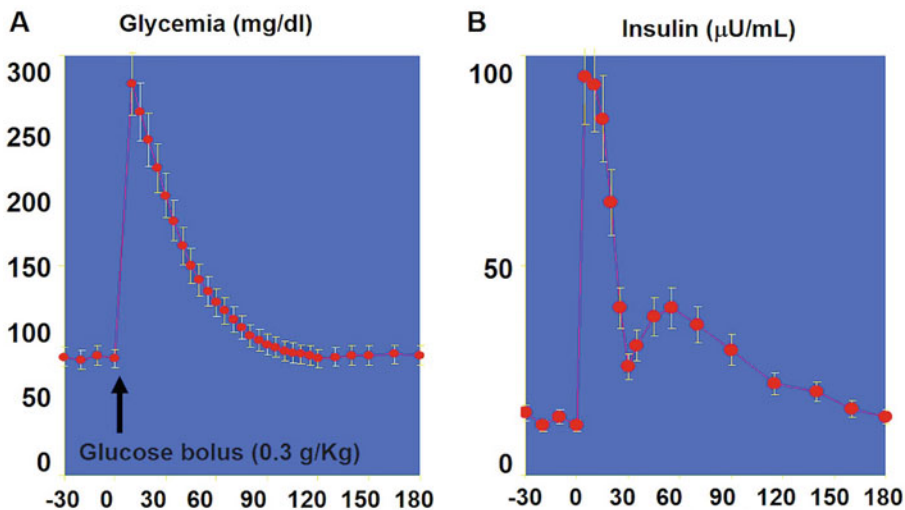


Fig. 1 Intravenous glucose test (IVGTT): (a) after an intravenous glucose bolus, (b) a biphasic response of insulin secretion is observed. The first-phase acute insulin response (AIR) is followed by a slower and more blunted secretion rise (second phase). Unpublished data

the acute insulin response (AIR), calculated from the insulin concentrations in the first 5–10 min of the test. There are different AIR formulas, including the area under the curve (AUC) of plasma insulin, the average or the sum of stimulated plasma insulin concentration, or the peak of plasma insulin concentration. In any case, the incremental values are preferred, that is, the change from the mean basal value. Optionally, C-peptide may be used instead of insulin to calculate the same indexes or calculation of the insulin secretion rate (ISR) using mathematical modeling (C-peptide deconvolution) [9].

The indexes of second-phase insulin secretion are based on the absolute or incremental areas under the curve (AUC) of insulin or C-peptide concentrations from 10 min (alternatively, from 30 to 60 min) to 180–240 min. The glucose curve must be taken into account, considering that it varies considerably in the second phase and that the insulin secretion depends directly on it. The most widely used index is the insulin AUC ratio (or C-peptide) to the glucose AUC.

It is assumed that the first phase of insulin secretion does not require adjustment to the glucose peak as the bolus dose is standardized. On the other hand, a cornerstone of the interpretation of AIR is its dependence on the prevailing insulin sensitivity. It might be less relevant in individuals with diabetes mellitus, in whom the AIR is virtually abolished, although there may still be a (small) acute response [11].

The frequently sampled IVGTT (FSIGT) is also useful to measure insulin sensitivity along with insulin secretion in a single test, using the software-assisted mathematical modeling (minimal model, MINMOD) [11, 13]. In order to improve the minimal model estimate of insulin sensitivity, intravenous insulin is administered 20 min after the glucose bolus, what is called the insulin-modified minimal model FSIGT. In this protocol, the interpretation of the second-phase insulin secretion may not be appropriate due to the presence of exogenous insulin, which may be partially overcome by the measurement of C-peptide (although some suppression of the endogenous insulin secretion by the exogenous insulin cannot be discarded).

The IVGTT duration is determined by the mathematical model based on the assumption that glucose concentration ends near the basal level. It is particularly relevant for individuals with diabetes, in whom it is expected to take longer to occur [11, 13]. The IVGTT may be shortened if only the first-phase insulin is needed (10-min protocol), usually when another test is chosen to evaluate insulin sensitivity (e.g., euglycemic-hyperglycemic clamp), which is conducted in a different day or 60 min after the IVGTT (Botnia clamp) [14].

2.1 Materials

1. Vein catheters.
2. Large-volume syringes (and a syringe pump, if possible).
3. 20–25% Glucose solution.
4. Regular insulin (100 UI/ml) (for insulin-modified IVGTT).
5. K⁺-EDTA blood collection tubes (for insulin and C-peptide).
6. Glucose tubes.
7. Ice slurry for lab tubes.
8. Optional (for bedside glucose analysis): glucose Analyzer (YSI 2700, YSI Life Sciences, Yellow Spring, OH, USA) and laboratory centrifuge and centrifuge tubes (for glucose analysis).

2.2 Methods

1. After an overnight fast (8–12 h), a catheter is inserted in an antecubital vein for blood sampling, and a second catheter is inserted in a contralateral antecubital vein for solution infusion. The patency of the venous accesses is maintained by slow saline drip.
2. At least two basal samples are drawn (15–30 min before and at time 0, immediately before glucose infusion) for the average basal measurements.
3. A 20–25% glucose bolus (0.3 g/kg body weight) is injected in a contralateral vein within 0.5–1 min, using a manual large-volume syringe, preferably >20 ml (or a syringe infusion pump, if available) (*see Note 1*).
4. After the glucose infusion, blood samples are drawn at frequent intervals over 180–240 min. In the minimal model (MIN-MOD) frequently sampled IVGTT (FSIGT), which is the most widely used protocol, the samples are drawn at 3, 4, 5, 6, 8, 10, 14, 19, 22, 25, 30, 40, 50, 60, 70, 80, 100, 140, and 180 min.
5. Blood samples for measuring insulin (and optionally, C-peptide) are put in K⁺-EDTA tubes and kept in an ice slurry, followed by immediate centrifugation and frozen storage or frozen transportation to the laboratory. The glucose tubes are used for laboratory glucose analysis immediately after the test; optionally, blood samples may be centrifuged immediately for testing in a bedside glucose analyzer.
6. In the insulin-modified minimal model FSIGT, an intravenous insulin dose (0.03–0.05 UI/kg) is administered 20 min after the glucose bolus, over 5 min (between 20 and 25 min).
7. At the end of the test, a meal is offered to the individual.

3 Hyperglycemic Clamp

The hyperglycemic glucose clamp assesses both first- and second-phase insulin secretion and simultaneously evaluates insulin sensitivity [4, 15]. The objective of the hyperglycemic clamp is to achieve a steady hyperglycemic state and a corresponding endogenous insulin response. The most used target glycemia is 180 mg/dl because it is in the range of (near) normal postprandial glycemia and near the threshold of urinary glucose excretion. The usual duration of the hyperglycemic clamp is 180 min (minimum 120 min). It may be longer in some protocols, usually in multi-stepped hyperglycemic levels and/or combined with other stimuli (standard meal or intravenous infusions of insulin secretagogues, such as incretins or arginine) [2, 4].

The first phase of insulin secretion is assessed in the initial 10 min, after an initial priming glucose dose. The first phase secretion index (acute insulin response, AIR) is similar to that obtained with the IVGTT and shares the same physiological interpretation (see the corresponding session). After that, a variable glucose infusion rate (GIR) is required to maintain constant hyperglycemia. In response, the plasma insulin concentration increases gradually (corresponding to the second-phase insulin secretion), and it is expected to plateau in the second or third hour of the test (Fig. 2). At this time, GIR is also expected to be stable (variation <5%). The variation in the glucose infusion rate is essentially dependent on both endogenous insulin production (and clearance) and insulin sensitivity.

The indexes of second-phase insulin secretion are calculated as the absolute or incremental areas under the curve (AUC) of insulin or C-peptide concentrations from 10 min after the start of glucose infusion up to the end of the test or in the last 60 min of the test. Alternatively, the average insulin or C-peptide concentrations in any of these time intervals may be used instead of AUC. In opposition to the IVGTT, the insulin response usually is adjusted to the glucose concentration, because by definition, it is clamped in the same level in all individuals.

The insulin sensitivity index is calculated as the average glucose infusion rate (GIR) during the last hour of the clamp, minus the occasional glucose urinary excretion, adjusted (divided) by the average plasma insulin concentration during the same interval [15]. Under stable conditions of constant hyperglycemia (third hour of the clamp), the amount of glucose infused (GIR) gives an estimate of the glucose disposal in the tissues since endogenous glucose production should be suppressed, similarly to what occurs during the hyperinsulinemic-euglycemic clamp (see the corresponding session below) [15]. The insulin sensitivity index of the hyperglycemic clamp correlates to that of the

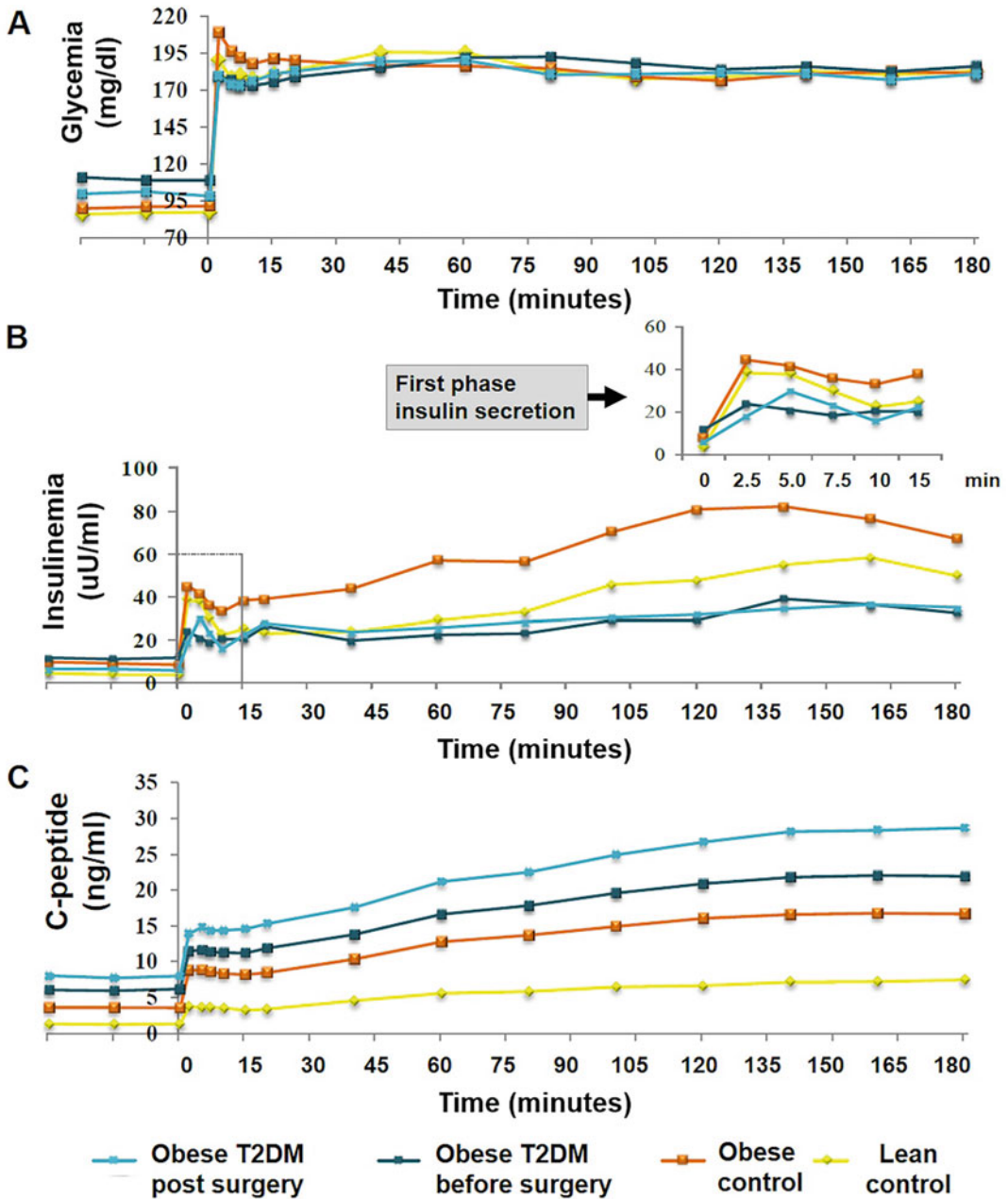


Fig. 2 Hyperglycemic clamp test applied to studying the effects of bariatric/metabolic surgery on glucose homeostasis. (a) Plasma glucose sharply raises and is kept at a constant hyperglycemic level, resulting in (b) a biphasic pattern of insulin secretion (first and second phases). First phase insulin secretion is typically blunted in type 2 diabetes (T2DM) but may recover after metabolic surgery and increases in obesity with normal glucose tolerance as an adaption to insulin resistance. (b, c) This adaptation of beta cells is also observed as an increase in second-phase insulin and C-peptide levels. Unpublished data

hyperinsulinemic-euglycemic clamp [15, 16]. However, the hyperglycemic clamp depends on endogenous insulin secretion, and it is not appropriate to study individuals with complete beta-cell failure, as is the case of type 1 diabetes. Comparing subjects with different glucose levels and/or beta-cell capacities (e.g., subjects with normal glucose tolerance and diabetes) require caution [4, 15].

C-peptide may be used to calculate the insulin secretion rate (ISR) in both first and second phases using C-deconvolution and other mathematical modeling methods to estimate beta-cell function and insulin sensitivity [9, 17, 18].

3.1 Materials

1. Heating pad (or warm chamber with heated air, if possible).
2. Vein catheters.
3. High-precision infusion pump.
4. Large-volume syringes (and a syringe infusion pump, if available).
5. 20–25% glucose solution.
6. Laboratory centrifuge and centrifuge tubes (for glucose analysis).
7. Glucose Analyzer (YSI 2700, YSI Life Sciences, Yellow Spring, OH, USA).
8. K⁺-EDTA blood collection tubes (for insulin and C-peptide).
9. Ice slurry (for storage of laboratory tubes).

3.2 Methods

1. After an overnight fast (8–12 h), the subject is kept lying on a bed in the supine position. A vein catheter is inserted in an antecubital vein for solution infusion. A second catheter is inserted in a contralateral dorsal hand or wrist vein, in a retrograde fashion, for blood drawing, and this hand is warmed to 50–70 °C using a heating pad, folded around the hand (or a warm chamber with heated air) to obtain arterialized venous blood (*see Note 2*). The patency of the venous accesses is maintained by slow saline drip.
2. At least two basal samples are drawn (15–30 min before and at time 0, immediately before glucose infusion) for the average basal measurements (*see Note 3*).
3. An infusion pump is installed to administer a 20–25% glucose solution in the antecubital vein throughout the test.
4. A priming glucose bolus is manually injected within 0.5–1 min, using a large-volume syringe (at least 20 ml), or using a syringe infusion pump (if available). This priming dose sharply raises the glucose concentration to the target level of hyperglycemia, usually 180 mg/dl (*see Note 4*). The formulas for the calculation of the glucose priming dose are empirical. One of the most used is the following [16]:

$$25\% \text{ glucose bolus dose (in ml)} = 4 \times \text{weight (in kg)} \times 1.5 \times [\text{target glycemia (in mg/dl)} - \text{basal glycemia (in mg/dl)}] \times 10^{-3}$$

For 50% glucose solution, the equation must be divided by 2. Other solution concentrations require proportional adjustments.

5. After the priming glucose bolus, the hyperglycemic target is maintained by a variable glucose infusion rate (GIR) during the remaining experiment period (usually 180–240 min), using an infusion pump. Blood is sampled frequently and centrifuged immediately for plasma glucose testing using a bedside glucose analyzer. Glucose levels are measured at 2.5-min intervals from zero to 10 min, and at intervals of 5 min (eventually, 10 min) from 10 to 180–240 min, and the glucose infusion rate is adjusted accordingly to maintain the hyperglycemic target (*see Note 5*). After each adjustment of GIR, plasma glucose concentration should be measured after an interval of 5 min (to permit stabilization of the glucose level in its distribution space), and further adjustments are performed as needed.
6. A steady state is reached in the third hour of the test when the plasma insulin concentrations are expected to plateau, and GIR should be stable (ideally, variation <5–10%) as well as the plasma glucose level (less than 10 mg/dl above or below the target).
7. Blood sampling for measuring insulin (and optionally, C-peptide) is performed at 2.5-min intervals from 0–10 min and intervals of 20 min from 10 to 180–240 min.
8. Blood samples are put in K⁺-EDTA tubes on ice, followed by immediate centrifugation and frozen transportation to the laboratory.
9. At the end of the test, a meal is offered to the subject, and bedside glucose levels are measured until 60 min after the end of glucose infusion (*see Note 6*).

4 Oral Load Tests: Oral Glucose Tolerance Test (OGTT) and Standard Mixed Meal Tolerance Test (MTT)

The oral glucose tolerance test (OGTT) is the reference method for the assessment of glucose tolerance, despite the poor reproducibility of the test (intra-subject variation of up to 50% for 2 h blood glucose) [19, 21]. In most cases, diabetes is diagnosed by random or fasting plasma glucose concentrations or glycosylated hemoglobin (HbA1c) without recourse to an OGTT. Patients with intermediate values of fasting glucose (impaired fasting glycemia) or HbA1c

should undergo OGTT. The test also has a particular place in diagnosing gestational diabetes mellitus, where fasting plasma glucose concentrations lack diagnostic sensitivity [19].

In clinical trials, the OGTT is used when the glucose tolerance classification is required. In research settings, it is a method to evaluate the beta-cell function, insulin sensitivity, and the incretin effect, based on the curves of glucose and insulin or C-peptide (Fig. 3); empirical mathematical formulas or more complex mathematical modeling are used for this purpose [5–7, 17]. Another research application of OGTT is the stimulation of gut hormones, although the mixed meal tolerance test (MTT) is usually preferred instead for this purpose (see below) [7].

The typical sampling of OGTT is done at 30-min intervals: zero (preload), 30, 60, 90, and 120 min after the glucose load. Two blood samples are enough for glucose tolerance classification: in the fasting state before the test and 120 min after starting. For gestational diabetes screening, an additional blood sample at 60 min is used [19–21].

The oral load tests typically last 120–180 min to evaluate insulin secretion and insulin sensitivity, but the duration may be up to 240–300 min. The blood collection intervals depend on the mathematical models that are chosen in the research protocol. In the first 30 min after baseline sampling, blood is drawn at 10, 20, and 30 min. Some reduced protocols evaluate 15 and 30 min, or only 30 min. After that, blood is drawn at 15–30 min until 180–240 min, and some extended protocols include a final blood collection at 300 min [5–7, 17].

Mixed meal tolerance tests (MTT) use a mixed meal instead of oral glucose, a more physiological stimulus than glucose alone. Although the MTT is not standardized for glucose tolerance classification and diabetes diagnosis, the combination of carbohydrates, lipids, and protein in mixed meals offer a stronger stimulus for secretion of gastrointestinal hormones, such as glucagon-like peptide 1 (GLP-1) and glucose-dependent insulintropic polypeptide (GIP) [17]. These hormones are responsible for the incretin effect, that is, the increment in insulin secretion after oral versus intravenous stimulus [7]. Even so, MTT and OGT have nearly the same recommendations, methods, and interpretation.

In the first studies, the mixed meals consisted of a standard meal (usually breakfast) with variable food content. Although still used, they have been extensively replaced by nutrition supplements, mainly ready-to-drink shakes, such as Ensure (Abbott), or a combination of shake and protein bars, used in our research laboratory. The macronutrient composition and calories vary across research protocols. Most standard mixed meals have at least 250–300 kcal and around 40–50 g of carbohydrates. Higher calorie and carbohydrate contents are preferred, although the available commercial products may limit this.

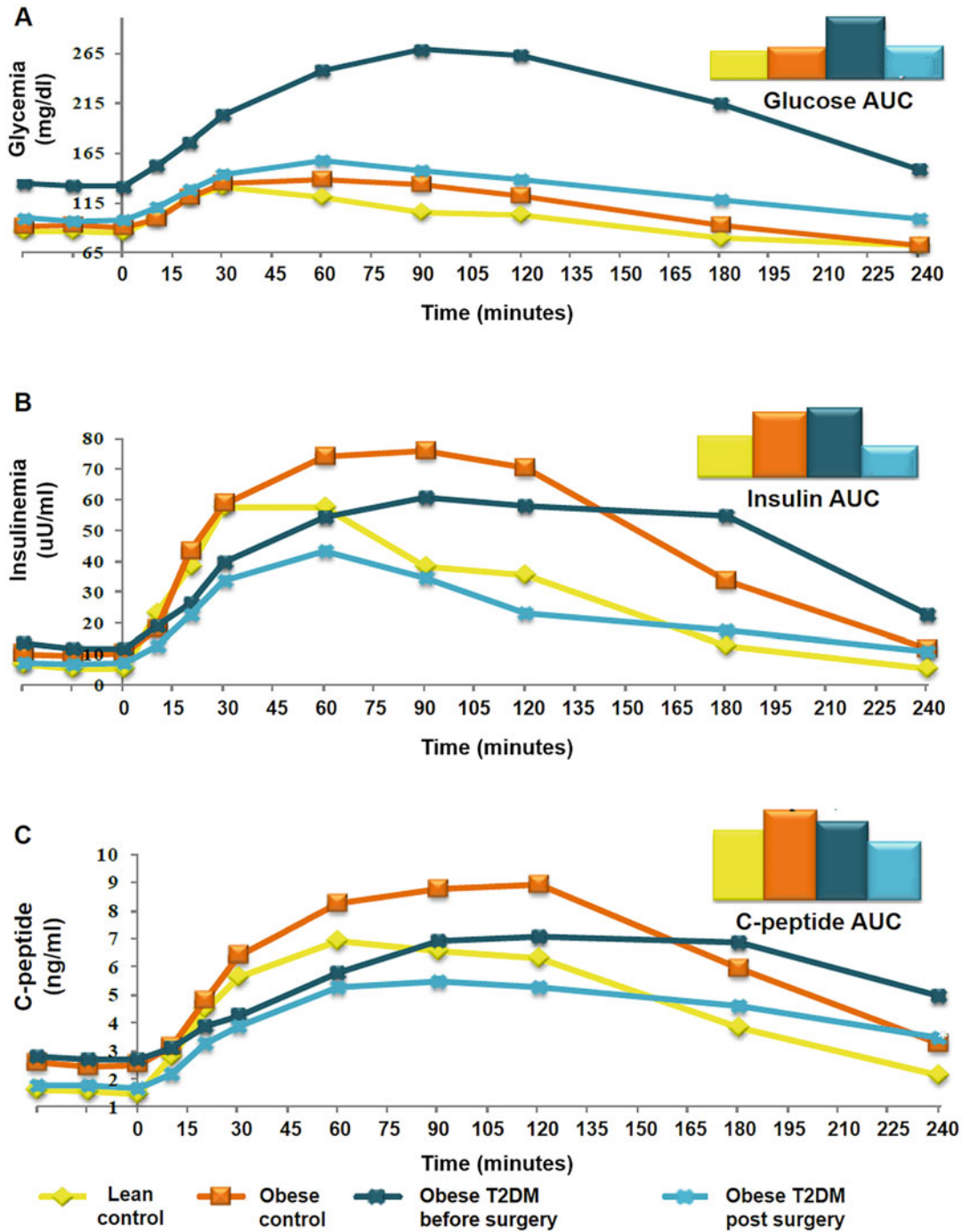


Fig. 3 Oral glucose tolerance test (OGTT) applied to studying the effects of bariatric/metabolic surgery on glucose homeostasis in the absorptive estate. (a) The typically increased plasma glucose in type 2 diabetes (T2DM) nearly normalizes after metabolic surgery despite lower levels of (b) insulin and (c) C-peptide, denoting improved insulin sensitivity. Unpublished data

We have standardized a combination of Ensure shake and a whey protein bar, resulting in 515 kcal (41.8% fat, 40.7% carbohydrates, and 17.5% protein) [22]. Many protocols use only liquid supplements such as Ensure High Protein (360 ml, 391 kcal, 8.5 g of fat, 44 g of carbohydrate, and 17 g of protein) or Ensure Plus (240 ml, 350 kcal, 11 g of fat, 48 g of carbohydrate, and 16 g of protein). An adjustment for body weight may be recommended (e.g., for pediatric population), usually 6 ml/kg body weight to a maximum of 360 ml (as for Ensure Plus, providing 1.1 kcal/ml; 23% fat, 55% carbohydrate, and 22% protein) [23].

The first 30 min of the oral load tests correlate to the first-phase insulin secretion, and the remaining time corresponds to the second phase [5–7]. The OGTT may be shortened if only the first phase is needed (30-min protocol). However, its interpretation is limited without adjustment to prevailing insulin sensitivity (as discussed IVGTT). In this case, another functional test should be used to evaluate insulin sensitivity (e.g., euglycemic-hyperglycemic clamp), which should be performed on a different day. A surrogate index of insulin sensitivity using basal (preload) parameters (e.g., HOMA-IR) may be acceptable.

The glucose appearance rate is not standardized and is unknown in oral load tests. It means that the insulin concentration and secretion must be adjusted to the prevailing glucose levels in both the first and second phases through empirical indices or mathematical modeling [5–7, 17].

The insulinogenic index is one of the most used, which is the ratio between the supra-basal increments at 30 min of insulin and glucose concentration: $[(30\text{-min insulin}/\text{basal insulin})/(30\text{-min glucose}/\text{basal glucose})]$. Similar indexes have been proposed, using 15-min values and/or replacing insulin for C-peptide, or using the ratio between the 30-min incremental area under the curve (AUC) of insulin (or C-peptide) and the incremental glucose AUC [5–7, 24, 25].

The ratio between the incremental areas under the insulin curve (or C-peptide) and glucose may be used as an index of second-phase or total insulin secretion. Empirical formulas have also been derived from intravenous glucose tests [24, 25]. Mathematical modeling, including C-peptide minimal models and C-peptide deconvolution, is based on complex beta-cell function aspects, such as insulin hepatic extraction, beta-cell delay time, and beta-cell sensitivity [17, 24–26].

Oral tests may be used to estimate insulin sensitivity through empirical formulas or complex mathematical modeling using C-peptide levels [27, 28]. The most used formulas are described below.

1. Matsuda's formula [29]: $ISI_{\text{comp}} = \sqrt{(G_b \bar{n} I_b \bar{n} G_m \bar{n} I_m)} / 1000$, where G_b and I_b are basal (preload) glucose and insulin,

respectively, and G_m and I_m are the mean concentrations during the OGTT (used sampling protocol: 0, 30, 60, 90, 120 min).

2. Stumvoll's Method [24]: $MCR_{est} = 18.8 - (0.27 \cdot BMI) - (0.0052 \cdot I_{120}) - (0.27 \cdot G_{90})$, where BMI (kg/m^2) is the body mass index, I_{120} is insulin at 120 min (pmol/l), and G_{90} is glucose at 90 min (mmol/l). This equation compares results with the last 60-min clamp-derived glucose clearance rate; thus, the units are thus $\text{ml}/\text{min}/\text{kg}$. Alternative formulas without BMI or for different sampling times have also been provided [24, 25].
3. OGIS Model [27]: $OGIS = f(G_b, G_{90}, G_{120}, I_b, I_{90}, D)$, where G and I are glucose and insulin concentrations at the time indicated by the subscript and D is the oral glucose dose (g/m^2 body surface area). The function (f) is derived from mathematical modeling and is calculated by software that may be accessed at <http://webmet.pd.cnr.it/ogis>. OGIS is a predictor of the clamp-derived glucose clearance rate adjusted to body surface area; thus, the units are $\text{ml}/\text{min}/\text{kg}$. Formulas for a 180-min OGTT are also available at the same web address.

4.1 Materials

1. Vein catheters.
2. 0.9% saline solution.
3. K^+ -EDTA blood collection tubes (for insulin and C-peptide).
4. Glucose tubes.
5. Ice slurry for lab tubes.
6. Optional (for bedside glucose analysis): laboratory centrifuge and centrifuge tubes (for glucose analysis); glucose Analyzer (YSI 2700, YSI Life Sciences, Yellow Spring, OH, USA).
7. Anhydrous glucose 75 g—for oral glucose tolerance test (OGTT).
8. Standard mixed meal (breakfast style or nutrition supplement, e.g., Ensure Plus or Ensure High Protein)—for standard mixed meal tolerance test (MMTT).

4.2 Methods

1. For 3 days before the test, the subject should be on an unrestricted weight-maintaining diet, with at least 150 g carbohydrate per day, and should exercise normally. On the day of the test, smoking should be avoided. Interpretation may be difficult in subjects taking β -blockers, diuretics, nicotinic acid, or high doses of glucocorticoids or during hospitalization or acute illness [20].
2. After an overnight fast (8–12 h), a vein catheter is inserted in an antecubital vein for blood sampling. The patency of the venous accesses is maintained by slow saline drip.

3. At least two basal samples are drawn (15–30 min before and at time 0, immediately before glucose load) for the average basal measurements (*see Note 6*).
4. The subject should remain seated to drink an oral load (75-g anhydrous glucose solution for OGTT, or standard mixed meal for MMTT) within 5 min (if not possible, within less than 10 min). After drinking, the participant should rest sitting or lying down.
5. The typical sampling of OGTT is done at 30-min intervals: zero (preload), 30, 60, 90, and 120 min after the glucose load. Two blood samples are enough for glucose tolerance classification: in the fasting state before the test and 120 min after starting. For gestational diabetes screening, an additional blood sample at 60 min is used. A 60-min collection has also been proposed for glucose intolerance screening in the general population.
6. The test lasts 180–300 min to evaluate insulin secretion and insulin sensitivity, although a 120-min duration may be used. The blood collection intervals depend on the mathematical models that are chosen in the research protocol. In the first 30 min, blood is drawn at 10, 20, and 30 min. Some reduced protocols evaluate 15 and 30 min, or only 30 min. After that, blood is drawn at 15–30 min until 180–240 min, and some extended protocols include a final blood collection at 300 min.

5 Insulin Tolerance Test (ITT)

The original concept of insulin sensitivity was derived from the empirical observation of heterogeneous requirements of exogenous insulin for lowering plasma glucose levels among individuals with insulin-dependent diabetes [1]. Thus, the glucose decay to a standardized insulin bolus seems to be an obvious method to measure insulin sensitivity. In the insulin tolerance test (ITT), a single intravenous insulin bolus provokes a fall in plasma glucose, and the glucose decay rate is used to estimate insulin sensitivity. During the test, plasma glucose quickly decreases to hypoglycemic levels. A short (15 min) protocol is preferred to avoid the confounding factor of hypoglycemia-induced counter-regulatory hormones (cortisol, catecholamines, growth hormone) that slow the glucose disappearance rate and for safety reasons [30].

The most used insulin sensitivity index is the constant (K) rate of glucose decay (Kitt). Kitt represents the percent decline in plasma glucose levels per min (%/min) and is determined by the ratio of $0.693/t^{1/2}$, where the denominator is the half-life of plasma glucose decay, calculated from the slope of the linear regression of the logarithm of blood glucose against time during the

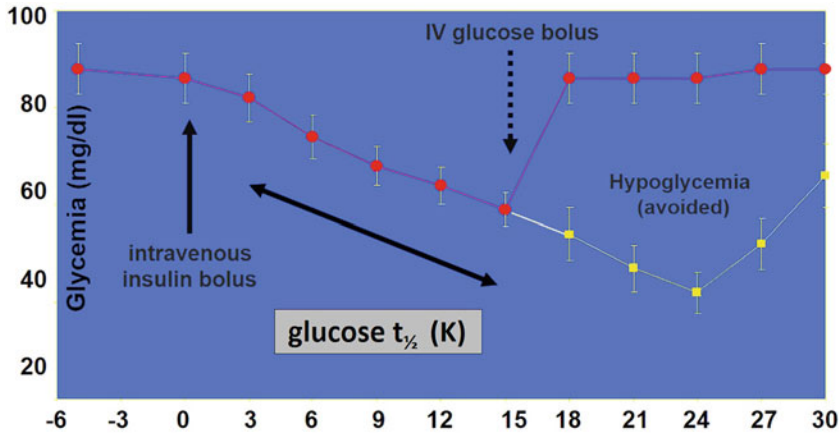


Fig. 4 Insulin tolerance test (ITT): the action of an intravenous insulin bolus on plasma glucose concentration is measured as the constant (K) rate of glucose decay (Kitt). The Kitt index represents the percent decline in plasma glucose levels per min, calculated from glucose half-life ($t_{1/2}$). The test is interrupted after 15 min in order to avoid hypoglycemia. Unpublished data

15 min interval (Fig. 4). An alternative insulin sensitivity index can be calculated by $\Delta G/G_b$, in which ΔG is the difference between baseline glycemia and the final glycemia (after 15 min), divided by baseline glycemia (G_b). Kitt has been validated against the hyperinsulinemic-euglycemic clamp [30]. However, it relies on a very short experiment interval that lacks a steady state, resulting in higher intra-individual variability in comparison to glucose clamps, so it may not be appropriate for small samples.

5.1 Materials

1. Vein catheter.
2. 0.9% saline solution.
3. 20–50% glucose solution.
4. Regular insulin (100 UI/ml).
5. 0.3–0.5 ml insulin syringe with needle.
6. Syringe without needle of 5-ml capacity or larger.
7. Glucose tubes.
8. Ice slurry for lab tubes.
9. Optional (for bedside glucose analysis): laboratory centrifuge and centrifuge tubes (for glucose analysis); glucose Analyzer (YSI 2700, YSI Life Sciences, Yellow Spring, OH, USA).

5.2 Methods

1. After an overnight fast (8–12 h), the subject is kept lying on a bed in the supine position. A vein catheter is inserted in an antecubital vein for insulin infusion and blood sampling. The patency of the venous accesses is maintained by slow saline drip.

2. At least two basal samples are drawn (5 min before and at time 0, immediately before insulin bolus infusion) for the average basal glucose measurements.
3. The regular insulin (0.1 U/kg) is prepared using a 0.3–0.5 ml insulin syringe (for dose precision of 1 IU) and transferred to a larger syringe with saline solution (*see Note 7*). The syringe is gently agitated, and the insulin solution is rapidly injected intravenously.
4. After the insulin bolus, blood is drawn at 3, 5, 7, 10, and 15 min for glucose measurement.
5. Immediately after the last blood sampling, the test is interrupted with a slow intravenous bolus of 50% glucose (20 ml) to reverse and prevent hypoglycemia. A meal is offered to the subject, and bedside glucose levels are measured until 30 min after the test.

6 Euglycemic-Hyperinsulinemic Clamp

The euglycemic-hyperinsulinemic clamp technique is traditionally recognized as the “gold-standard” method for assessing insulin sensitivity in vivo [1, 4]. This status is attributable to some technical advantages and the extensive knowledge about the pathophysiology of carbohydrate metabolism derived from glucose clamp studies.

The concept of insulin sensitivity extends to all actions elicited by insulin signaling, including protein synthesis, lipolysis inhibition, activation of growth pathways, among others. However, insulin sensitivity in vivo usually refers to glucose metabolism because of the tight relationship between plasmatic glucose and insulin [1].

One of the main advantages of the euglycemic-hyperinsulinemic clamp is the high reproducibility (intra-individual coefficient of variation of ~10%), which is mostly justified by a long-duration steady-state condition [31]. Other major advantages are the independence of endogenous insulin secretion and its simple conceptual principles (described below), which allow protocol versatility and straightforward interpretation of the experiment results [31]. It does not mean that the measurement of insulin sensitivity from the clamp test is superior to those estimated by other techniques, such as the intravenous glucose tolerance test, and the choice of one method over others is made primarily based on practical issues, including researchers' expertise, available resources and sample size [1, 31]. Despite its complex execution, the main concepts of the euglycemic-hyperinsulinemic clamp are quite simple (Fig. 5). In the normal fasting (postabsorptive) state, glucose homeostasis is maintained in a closed loop in which a relatively low insulin secretion tightly regulates the endogenous glucose output (~95% from the liver).

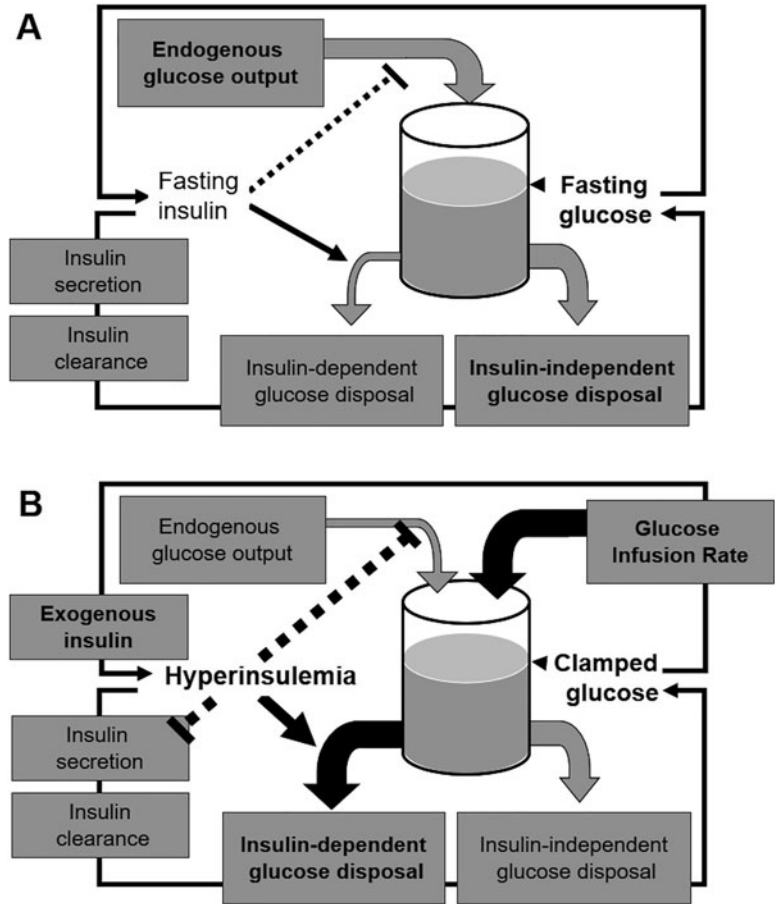


Fig. 5 Schematic diagram of (a) normal fasting glucose homeostasis represented in a closed loop. The glucose influx to the glucose space (extracellular fluid) equals the glucose outflux, resulting in constant glycemia. The only source of glucose input comes from endogenous glucose output (mostly from the liver and from kidneys), and the glucose disposal is predominantly insulin-independent. (b) The closed-loop is “opened” during the hyperglycemic euglycemic clamp test by a hyperinsulinemic plateau achieved through exogenous insulin infusion, while the endogenous insulin secretion is suppressed. Hyperinsulinemia almost completely suppresses endogenous glucose output, and the exogenous glucose infusion accounts for insulin-dependent, whole-body glucose requirements under these conditions. (Dashed lines in the figure represent suppression)

In contrast, the glucose uptake is mostly independent of insulin action and prevails in insulin-independent tissues (e.g., the brain) (Fig. 5a). In the clamp study, this loop is opened through a constant infusion of exogenous insulin to produce a hyperinsulinemic plateau that suppresses most of the endogenous glucose output and increases the whole-body glucose disposal (~90% in the skeletal muscles), which would result in a decline of plasma glucose concentration.

A second intervention prevents this decline in the loop: an exogenous infusion of glucose that exactly matches the insulin-dependent glucose uptake out of the glucose space (extracellular fluid) into the tissues as well as the reduction of endogenous glucose output (Fig. 5b) [1, 4, 31].

It is impossible to measure the in vivo glucose utilization in the tissues directly in the clamp experiment. However, the amount of exogenous glucose required to maintain constant euglycemia during a hyperinsulinemic state equals the amount of glucose that is taken up from the glucose space to be metabolized; thus, the glucose infusion rate (GIR) is a measurable variable that corresponds to the insulin-stimulated whole-body glucose disposal. For this reason, GIR is the primary insulin sensitivity index of the clamp test, expressed as $\text{mg} \cdot \text{kg body weight}^{-1} \cdot \text{min}^{-1}$. It is most frequently called the “*M*-value,” as it refers to the “metabolized glucose,” after mathematical adjustments (see below) [1, 4].

The *M*-value represents the whole-body glucose disposal rate at a specific level of hyperinsulinemia. As with other dynamic studies (despite acknowledging as “gold standard”), there are no broadly accepted cut-off values to define “insulin resistance” or “low insulin sensitivity” for the glucose clamp, and age- and weight-matched control groups are required in the protocols. In large samples, insulin resistance may be considered in the lower quartile of the *M*-value in a heterogeneous or nonselected population [1]. *M*-values below the 95% confidence interval of a healthy, glucose tolerant population may be considered, which grossly would give a cut-off around $5 \text{ mg} \cdot \text{kg body weight}^{-1} \cdot \text{min}^{-1}$, based on a series of previous studies using the insulin infusion of $40 \text{ mU}/\text{m}^2/\text{min}$ [1].

The hyperinsulinemic-euglycemic clamp technique is sustained by some assumptions that must be fulfilled.

One assumption is that endogenous glucose output is suppressed or negligible during the clamp. As a result, the amount of glucose infused equals the amount of glucose metabolized in peripheral tissues, mainly in the skeletal muscles, giving an index of peripheral insulin sensitivity essentially [4]. However, GIR corresponds to the net effect of insulin on whole-body glucose metabolism in which the endogenous glucose output may not be completely inhibited in some clinical conditions, depending on the hyperinsulinemic level. Therefore, the variable measured in the hyperinsulinemic-euglycemic clamp is more appropriately called a whole-body insulin sensitivity index. The distinction between the hepatic and the peripheral insulin sensitivities requires a tracer-dilution technique with radioisotope-labeled glucose during the hyperinsulinemic-euglycemic clamp. The hepatic glucose production is determined from the difference between tracer-derived glucose disposal and the known exogenous glucose infusion rate in the fasting state, followed by the hyperinsulinemic state

during the glucose clamp. The hepatic insulin sensitivity is calculated as the degree of suppression of the hepatic glucose production and the peripheral insulin sensitivity as the difference between the hepatic and the whole-body insulin sensitivity [1, 31, 32].

A second assumption is the achievement of a stable hyperinsulinemic plateau through a constant intravenous infusion of exogenous insulin. This condition means that the hyperinsulinemic-euglycemic clamp does not depend on pancreatic insulin production, and the method is appropriate in a full range of beta-cell function [1, 4]. This is a major advantage of this method over others to assess insulin sensitivity. The suppression of beta-cell secretion induced by the hyperinsulinemic state is combined with the euglycemic condition that also prevents the stimulation of endogenous insulin secretion that otherwise could alter the desired plasma insulin level, and the hyperinsulinemic state suppresses insulin [1].

A full range of hyperinsulinemic targets have been tested (from ~10 to 1000 mU/m²/min), including multistep hyperinsulinemic clamps. However, the relationship between insulin sensitivity is not linear and tends to plateau [1]. The standard insulin infusion rate during a hyperinsulinemic-euglycemic clamp test is 40 mU/m²/min¹, which corresponds approximately to 1 mU/kg/min. The resulting steady plasma insulin concentrations are between 70 and 130 mU/ml [1], within the range of physiological postprandial hyperinsulinemia [33]. This range serves for most research purposes due to a good balance between suppression of endogenous glucose output (which may not be achieved at lower insulin levels) and discrimination of small differences in peripheral insulin sensitivity (which may disappear at higher levels) [1].

The variation in the plasma insulin concentrations for the same insulin infusion rate among individuals or different studies may relate to differences in insulin clearance, fasting plasma insulin concentrations, suppressing endogenous insulin secretion, and even to different insulin assays [1].

A third assumption is the maintenance of constant euglycemia, usually convened as 90 mg/dl (or eventually 100 mg/dl), with a narrow variation around this goal. In some protocols, the individual fasting plasma glucose concentration is clamped, which is termed an isoglycemic hyperinsulinemic clamp. However, the same glycemic goal should be established for all individuals for comparison. Individuals with fasting hyperglycemia should be corrected during the clamp to reach the predetermined euglycemic level.

Finally, another assumption is the achievement of a steady state in which the glucose infusion rate (GIR) and glycemia are constant (variation <5–10%) (Fig. 6). The duration of the clamp test must be long enough to reach this condition for at least 30 min, taking into account that there is considerable variation in glycemia (and consequently in GIR) before reaching the steady state [1, 4].

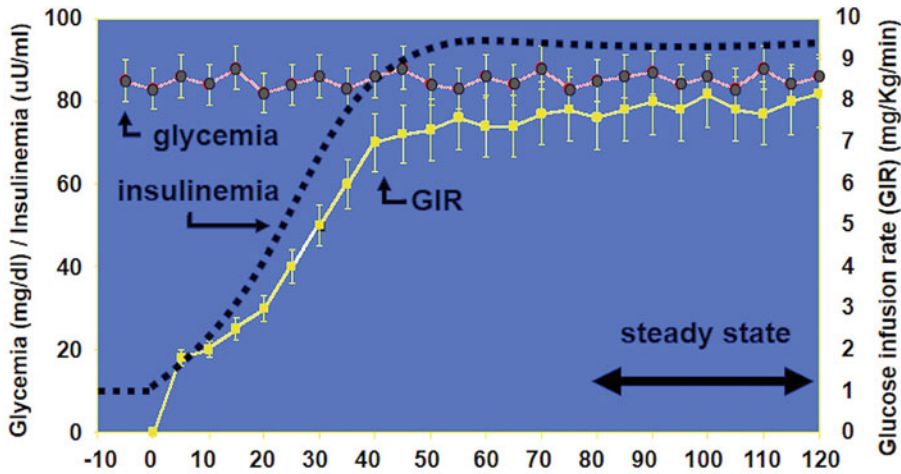


Fig. 6 Curves of plasma glucose and insulin and glucose infusion rate (GIR) during the hyperinsulinemic-euglycemic clamp test. A steady state must be observed in the final 30–60 min of the experiment

In healthy, nonobese individuals without significant metabolic disorders, a clamp test duration of 120 min may be adequate; the steady state is usually reached in the second hour, and the time periods of 60–120 min and 80–120 min are the most used for calculation of the insulin sensitivity index [31]. However, in individuals with dysmetabolic conditions like obesity and diabetes mellitus, a steady state usually will not occur before the third hour, and the time periods of 120–180 or 150–180 min are used. A progressive increase in glucose disposal is generally observed as the duration of the clamp test increases, resulting from increases in glucose oxidation [34]. Thus, the comparison of M -values between individuals or groups requires that the same time periods are used to calculate the M -values. Therefore, the duration of the clamp test in a study is usually predefined as the least duration that all participants are expected to achieve the steady state (120 or 180 min).

Even in a steady state, plasma glucose levels are not always perfectly constant. The GIR must be adjusted to changes in glycemia that do not represent real glucose uptake using a factor called “space correction” [4]. It is the rate at which glucose was added to or removed from the glucose space independently of changes in glucose uptake.

The M -value is calculated considering the space correction: $M = \text{GIR} - \text{SC} - \text{UC}$, where GIR is the glucose infusion rate, SC the space correction, and UC the correction for urinary glucose loss (rarely used because glycosuria is negligible in euglycemia). The M -value is calculated for each period of 10–20 min during the test, and the final index is the mean of M -values during 30–60 min intervals in a steady state. The space correction is calculated as follows: $\text{SC} (\text{mg/kg/min}) = (G_2 - G_1) \cdot 0.095$, where G_2 and G_1 are the

plasma glucose concentrations (mg/dl) at the end and the beginning of each 20-min period, respectively. If 10-min periods are used, the result must be multiplied by 2; for any other time periods, the equation must be adjusted. The number 0.095 derives from calculations that comprise the whole-body distribution volume of glucose expressed in liters [0.19 (l/kg body weight)]. The space correction will perform accurately only if glycemia is within a narrow range of the target level (± 10 mg/dl). In addition, the time interval between the two measurements of plasma glucose concentrations used for the calculation of the space correction should not exceed 20 min. For any other time interval, the equation must be adjusted.

Further adjustments of the M -value are advisable for comparisons. As described above, the whole-body glucose disposal is usually presented per kg body weight ($\text{mg} \cdot \text{kg body weight}^{-1} \cdot \text{min}^{-1}$). The adjustment of M -values to fat-free mass (MFFM) is the most recommended [1, 31]. This correction accounts for gender-related differences in fat mass [8] and prevents underestimating insulin sensitivity in obesity [35]. Other adjustment factors are not usual, such as the resting energy expenditure (MREE) or the steady-state glycemia during the clamp (which yields the metabolic clearance rate, MCR) [1, 31]. The correction for steady-state plasma insulin concentration during the clamp (M/I) seems intuitive, but it adds no benefit and increases the dispersion of values [1].

6.1 Materials

1. Heating pad (or warm chamber with heated air, if possible).
2. Vein catheters and plastic three-way stop cock.
3. High-precision volumetric infusion pump and respective infusion set.
4. High-precision syringe infusion pump with fine adjustment of the infusion rates (0.02–0.15 ml/min).
5. Large-volume (50–60 ml) syringe (compatible with the syringe infusion pump).
6. Long extension tube (for connection between syringe pump and vein catheter).
7. Extension tube (for venous access).
8. 20–25% glucose solution bag.
9. 0.9% saline solution.
10. Regular insulin (100 UI/ml).
11. 0.3–0.5 ml insulin syringe with needle.
12. Laboratory centrifuge and centrifuge tubes (for glucose analysis).
13. Glucose Analyzer (YSI 2700, YSI Life Sciences, Yellow Spring, OH, USA).

14. K⁺-EDTA blood collection tubes (for insulin and C-peptide).
15. Ice slurry (for storage of laboratory tubes).

6.2 Methods

1. For insulin infusate preparation, a large-volume syringe is filled in with 0.9% saline solution, completing the volume with 2 ml of the individual's blood, added to every 48 ml (for a total of 50 ml). The blood prevents insulin adsorption to plastic surfaces of the syringe and infusion lines, particularly with long extension lines. Then the regular insulin is added using a 0.3–0.5 ml insulin syringe (for dose precision of 1 IU), and the syringe is gently agitated before installation in the infusion pump. The most widely insulin infusion rate is 40 mU/m²/min, proposed in original protocols [1, 4] (*see Note 8*).

To simplify preparation, the infusate volume and the insulin pump infusion rate are predefined, and the insulin dose is calculated as follows:

$$\text{Insulin dose (IU)} = [\text{Ins (40 mU/m}^2\text{/min)} \bar{n} \text{BSA (m}^2\text{)} \bar{n} 60 \text{ min } \bar{n} \text{Syringe Vol. (50 ml)}] \bar{n} [\text{Pump Infusion Rate (11.4 ml/h)} \bar{n} 1000]^{-1}$$

Ins represents insulin infusion rate (per minute), usually adjusted for body surface area (BSA, in square meters). The Syringe Vol. refers to the filled volume in the syringe. The Pump Infusion Rate is the predefined constant rate of an insulin infusion pump (ml/h), here chosen to be 11.4 ml/h. The number 1000 in the denominator converts the insulin units from mU to IU [4] (*see Note 9*).

2. After an overnight fast (8–12 h), the subject is kept lying on a bed in the supine position. A vein catheter is inserted in an antecubital vein for solution infusion. In order to avoid possible interference of ipsilateral infusion and blood sampling, a second catheter is inserted in a contralateral dorsal hand or wrist vein, in a retrograde fashion, for blood drawing.
3. The hand used for blood collection is warmed to 50–70 °C using a heating pad, folded around the hand (or a warm chamber with heated air) to obtain arterialized venous blood (*see Note 2*). The patency of the venous accesses is maintained by slow saline drip.
4. At least two basal samples are drawn (15–30 min before and at time 0, immediately before glucose infusion) for the average basal measurements.
5. An infusion pump is installed to administer a 20–25% glucose solution in the antecubital vein throughout the test (*see Note 10*), and another pump (syringe infusion pump) is installed in parallel in the same vein (using a three-way stopcock) to administer the insulin solution.

Table 1
Simplified protocol of primed insulin infusion for the hyperinsulinemic-euglycemic clamp test (40 mU/m²/min)

Time (min)	Insulin per body surface are per min (mU/m ² /min)	Infusion rate (ml/h)
0–3	160	45.6
3–7	80	22.8
7–end of test	40	11.4

6. In order to achieve and maintain a constant hyperinsulinemic state, a priming insulin infusion is administered in the first 7–10 min of the test, which acutely raises the plasma insulin to achieve a whole-body saturation of insulin receptors (*see Note 11*). The priming is performed in a declining fashion (Table 1) and is followed by a predefined continuous insulin infusion rate to maintain the desired hyperinsulinemic level (*see Note 12*). A steady-state insulin concentration is expected after 60 min.
7. During insulin priming (first 7–10 min), the glucose infusion is started following the same time schedule, following an empirical scale of low (though progressive) glucose infusion rates (GIR), which is suitable for insulin-resistant individuals or correction of mild fasting hyperglycemia (~100 mg/dl). Typically, GIR (mg/dl glucose per kg body weight per min) is gradually increased from 0.5 (0–3 min) to 1.0 (3–7 min) and to 1.5 (7–10 min). This protocol should be scaled up for individuals perceived as more insulin sensitive (eumetabolic, physically very active), starting with GIR >1–2 mg · kg⁻¹ body weight · min⁻¹. The glucose infusion should not start before reaching euglycemia for higher plasma glucose concentrations (up to ~150 mg/dl) (*see Note 13*). An electronic spreadsheet for converting glucose infusion rate (GIR) to volume infusion rate (Fig. 7) may be filled out before the procedure starts, inputting an individual's weight and glucose solution concentration. This is useful to streamline the infusion rate adjustments in the pump.
8. Plasma glucose levels are measured at 5-min intervals until the end of the test using a bedside glucose analyzer (*see Note 14*) to adjust the glucose infusion rate to achieve and maintain euglycemia (90 mg/dl). Ten-min intervals may be applied in some periods of the test. After each adjustment of GIR, plasma glucose concentration should be measured after an interval of 5 min (to permit stabilization of the glucose level in its distribution space), and further adjustments are performed as

GIR	Infusion rate (ml per hour)	GIR	Infusion rate (ml per hour)
0.5		6.0	
1.0		6.5	
1.5		7.0	
2.0		7.5	
2.5		8.0	
3.5		8.5	
4.0		9.0	
4.5		9.5	
5.0		10.0	

Fig. 7 Example spreadsheet for conversion of glucose infusion rate (GIR) to volume infusion rate for glucose clamp tests, according to body weight and the weight per volume of the glucose solution. Infusion rate = GIR (mg glucose per kg body weight per min) · body weight (kg) · [glucose weight per volume (mg/ml)]⁻¹ · 60 min, where the glucose weight per volume is usually 200 mg/ml (Glucose 20%) or 250 mg/ml (Glucose 25%)

needed. As a result, the glucose measurements are not always performed at exact 5-min intervals. On the other hand, a delay between blood collection and the respective adjustment of GIR may compromise the achievement of a steady state during the clamp test. For this reason, the glucose analyzer must be fast enough to provide measurements within less than a minute from blood sampling. A worksheet helps to track measurements and adjustments during the experiment (Fig. 8).

9. Although there are some proposed algorithms for the adjustment of GIR, the empirical adjustment based on the researcher's experience usually works better and is preferred. A general rule is to increment or decrement GIR by $0.5 \text{ mg} \cdot \text{kg}^{-1} \text{ body weight} \cdot \text{min}^{-1}$ whenever the glucose level is more than 5% below or above the target, respectively (*see* **Notes 15** and **16**).
10. A steady state is reached in the second or third hour of the test when the variation of GIR should be ideally less than 5–10% for a time period of at least 30 min at the end of the test. At this time, the variation of glycemia should be less than 5–10% above or below the goal (90 mg/dl).

Time (minutes)	Glycemia (mg/dl)	GIR	Infusion rate (ml/hour)	Time (minutes)	Glycemia (mg/dl)	GIR	Infusion rate (ml/hour)
- 60				85			
- 30				90			
- 15				95			
0		0.5		100			
3		1.0		105			
7		1.5		110			
10				115			
15				120			
20				125			
25				130			
30				135			
35				140			
40				145			
45				150			
50				155			
55				160			
60				165			
65				170			
70				175			
75				180			
80							

Fig. 8 Example worksheet for documentation of glycemia and glucose infusion rate (GIR) during glucose clamp tests. Typically, GIR is gradually increased in the first 7–10 min; then, it is empirically adjusted according to plasma glucose concentration, measured at 5-min intervals until the end of the test. Insulin-sensitive individuals will require higher starting rates

11. Blood sampling for insulin is performed at 30-min intervals or more frequently during the test. The plasma insulin analysis generally is not needed for interpretation of the results, but it is useful to evaluate the quality of the procedure, that is, the achievement of the desired constant hyperinsulinemic state, particularly in the second and third hours. C-peptide may be measured in the same schedule for estimating the suppression of endogenous insulin secretion. Blood samples are put in K⁺-EDTA tubes on ice, followed by immediate centrifugation and frozen transportation to the laboratory.
12. At the end of the test, the insulin infusion pump is turned off. A meal is offered to the subject and bedside glucose levels are measured until 60 min after the test. In order to prevent hypoglycemia, it is warranted to keep the glucose infusion while waiting for exogenous insulin clearance (~30 min, and up to 60 min) and the glycemc meal load (*see Note 17*).

7 Conclusion

The full comprehension of beta-cell function complexity requires different tests and the assessment of insulin sensitivity, which can be evaluated simultaneously with insulin secretion by most methods. In contrast to insulin sensitivity, which has the hyperinsulinemic-euglycemic clamp recognized as the “gold-standard” method, no single beta-cell test fulfills such status, and each one has its advantages and limitations. The possibility of concomitant assessment of insulin sensitivity during procedures primarily dedicated to evaluating insulin secretory response is an advantage. The hyperglycemic clamp, followed by IVGTT, typically represents this possibility. Both intravenous tests have in common standardized insulin secretory stimuli, meaning that interpretation of insulin response is less dependent on mathematical modeling, even though IVGTT does have a nonstandardized portion that depends on modeling for accurate interpretation of second-phase insulin secretion and insulin sensitivity. Nonstandardization of stimuli (most related to unpredictable and variable glucose concentrations) is a typical limitation of oral tests, and then they require complex mathematical interpretation. In turn, they are closer to normal living and enclose the insulin potentiation exerted by gut hormones and are useful in studying glucose intolerance, pharmacological and nutrition therapies, and food science.

The selection of the methods of assessment of glucose homeostasis in the research settings depends on the aspects of beta-cell function and insulin sensitivity that are relevant to the proposed hypothesis, the possibility of assessment of both beta-cell function and insulin sensitivity, and the accuracy of different methods according to the type of interpretation (such as empirical versus mathematical modeling), the sample size and the studied population. It also comprises the experiments' complexity and feasibility, taking into account the available laboratory equipment and computational resources, the previous experience of the researchers, and the possibility of cooperation with other research centers, particularly for the application of mathematical models.

8 Notes

1. Particular attention must be paid to the duration of the glucose bolus because of the narrow interval until the first blood sampling in IVGTT. Some protocols use a higher glucose bolus (0.5 g/kg of body weight), resulting in a larger volume (particularly in individuals with obesity), making it more to infuse the bolus within 1 min. With higher doses, plasma glucose concentrations may take longer to normalize, and they may

not return to basal levels within the specified duration of the experiment, mainly in individuals with diabetes mellitus.

2. The “heated hand technique” for venous blood arterialization is proposed to avoid differences in plasma glucose related to its uptake in distal arm tissues. It may not be a major concern in most cases, and this technique has been considered unnecessary by some authors. However, tissue glucose uptake is higher in insulin-sensitive individuals than in insulin-resistant ones, resulting in arteriovenous differences proportional to insulin sensitivity [1]. Therefore, the venous glucose clamping may have a little but not negligible negative impact in the determination of insulin sensitivity, particularly for comparison of heterogeneous groups.
3. In individuals with diabetes mellitus, basal hyperglycemia near or above the target glucose level may contraindicate the hyperglycemic clamp. In contrast to the euglycemic-hyperinsulinemic clamp, it is not desirable to bring the subjects to euglycemia through intravenous insulin infusion because this would confound the endogenous insulin.
4. The rapid bolus protocol in the hyperglycemic clamp reproduces the shape of the sharp pattern of first-phase insulin secretion obtained in the intravenous glucose tolerance test (IVGTT). The difference between the hyperglycemic clamp and the IVGTT is that the target glucose level is predicted to calculate the glucose bolus in the former but not in the latter. Other priming protocols are based on the classical description of the hyperglycemic clamp [4] and use a priming dose over 15 min in which half of the total glucose mass is infused during the first 5 min and the remainder during the next 10 min.
5. Although there are some proposed algorithms for the adjustment of GIR, the empirical adjustment based on the researcher’s experience usually works better and is preferred [1]. In the hyperglycemic clamp, a general rule is to increment or decrement GIR by $\sim 1 \text{ mg} \cdot \text{kg body weight}^{-1} \cdot \text{min}^{-1}$ whenever the glucose level is more than 5–10% below or above the target, respectively.
6. In clinical settings, oral load tests would not be recommended for subjects with fasting plasma glucose $\geq 126 \text{ mg/dl}$, although it is acceptable in research settings. However, the interpretation of the metabolic responses may be compromised as the basal glycemia approaches 200 mg/dl and definitively should not be performed with fasting plasma glucose $\geq 200 \text{ mg/dl}$. Glucotoxicity may lead to underestimation of insulin response. On the other hand, higher glucose levels after the oral load may increase the glucose effectiveness, that is, increased glucose concentrations promote its transfer to tissues independently of insulin action, thus overestimating insulin sensitivity.

7. It is advisable to dilute an amount of insulin that is higher than the calculated dose to improve the precision of insulin dosing in insulin tolerance test (ITT), usually in a ratio of 1 UI of insulin per 1 ml of saline solution.
8. In the hyperinsulinemic-euglycemic clamp, the insulin infusion rate may be calculated per kg body weight instead of per body surface area. The frequently used insulin infusion rate of 40 mU per square meter body surface area per minute [1, 4] corresponds to 1 mU per kg body weight per minute. However, calculation per body weight may not be appropriate in samples with different weight ranges or for which significant weight changes are expected, leading to heterogeneous hyperinsulinemic levels.
9. The 50-ml volume of insulin infusate is enough for a 180-min euglycemic hyperglycemic clamp. In protocols of longer duration, however, sequential infusates may be required. Preparing one stem infusate for the whole test before it starts is preferable to guarantee identical insulin concentrations throughout the test.
10. Glucose solutions with concentrations below 20% are not recommended for the hyperinsulinemic-euglycemic clamp because they may cause overhydration during the clamp test.
11. Unprimed insulin infusion protocols are generally not applied because it takes longer to achieve an appropriate plateau of insulin concentration, and there may not be enough time of steady state within the specified duration of the test.
12. Hyperinsulinemia may induce hypokalemia through stimulation of cellular uptake of potassium, and the serum potassium levels tend to decrease dose-dependently, as plasma insulin concentration gradually rises in the first 2 h of the clamp test [36]. However, the hyperinsulinemic levels are similar to those commonly reached in the postprandial state, and serum potassium concentration spontaneously reincreases in the third hour of the clamp [36]. Although some researchers recommend repeated measurement of plasma potassium or even concomitant prophylactic potassium infusion during the clamp test, most recognize that the serum potassium decrease is not clinically relevant, and usually, no specific measures are performed.
13. If the basal glycemia is above 150 mg/dl before the hyperinsulinemic-euglycemic clamp, it may take over an hour to normalize following the start of the protocol-specified insulin infusion. Therefore, it is advisable to reschedule the procedure in most cases because a delay to achieve the steady state may compromise the results. In case of hyperglycemia before or during the glucose clamp, an intravenous insulin bolus is not recommended to reach euglycemia because it would affect the

insulin levels. Even if the researchers wait at least 1 h for plasmatic clearance of the exogenous insulin, changes in insulin signaling (saturation of insulin receptors, sensitization), some suppression of endogenous glucose output, or the endocrine pancreas cannot be ruled out.

14. Laboratory glucose analyzers are recommended due to higher precision for bedside measurements during functional tests. However, they require careful maintenance and frequent calibration and may fail. Although not as precise, a backup portable glucometer may help continue the clamp test until any problem is resolved. Choose the most precise glucometer model and test the same blood sample twice (double-check).
15. Particular attention is recommended in the adjustment of GIR following the first 30 min of the clamp test when an increase in glucose disposal is usually noticed to avoid hypoglycemia. For the same reason, plasma glucose levels above the target may require less correction in the first ~30 min.
16. During the hyperinsulinemic-euglycemic clamp, a greater adjustment of GIR (increment or decrement of $1.0 \cdot \text{kg}^{-1} \cdot \text{body weight} \cdot \text{min}^{-1}$) may eventually be necessary (usually when glycemia is more than 10% off the target), and in case of hyperglycemia ($>100 \text{ mg/dl}$) during the procedure, the glucose infusion pump may be turned off temporarily.
17. After the end of the hyperinsulinemic-euglycemic clamp, the glucose infusion rate may be gradually decreased, usually by half, since insulin infusion ends and the meal starts, then again every 10–15 min, for at least 30–45 min.

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