Video Article

Characterization of Metabolic Status in Nonhuman Primates with the Intravenous Glucose Tolerance Test

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Abstract

The intravenous glucose tolerance test (IVGTT) plays a key role in the characterization of glucose homeostasis. When taken together with serum biochemical profiles, inclusive of blood glucose levels in both the fed and fasted state, HbA1c, insulin levels, clinical history of diet, body composition, and body weight status, an assessment of normal and abnormal glycemic control can be made. Interpretation of an IVGTT is done through measurement of changes in glucose and insulin levels over time in relation to the dextrose challenge. Critical components to be considered are: peak glucose and insulin levels reached in relation to T0 (end of glucose infusion), the glucose clearance rate *K* derived from the slope of rapid glucose clearance in the first 20 min (T1 to T20), the time to return to glucose baseline, and the **area under the curve (AUC).** These IVGTT measures will show characteristic changes as glucose homeostasis moves from a healthy to a diseased metabolic state[5]. Herein we will describe the characterization of nonhuman primates (Rhesus and Cynomolgus macaques), which are the most relevant animal model of Type II diabetes (T2D) in humans and the IVGTT and clinical profiles of these animals from a lean healthy, to obese dysmetabolic, and T2D state [8, 10, 11].

Video Link

The video component of this article can be found at https://www.jove.com/video/52895/

Introduction

The IVGTT is a convenient functional assay that is routinely used to determine the β-cell function in humans at different metabolic states [5, 7]. In animal models of T2D, it is well recognized as a tool to characterize animals that show metabolic disease progression from a healthy to a dysmetabolic hyperglycemic state [8, 9]. The closest animal model of T2D is demonstrated in nonhuman primates (NHPs), of which rhesus and cynomolgus macaques are notable examples. These animals naturally develop T2D with the same risk factors of age and obesity contributing to its incidence as in humans [10]. Furthermore, there is a similar disease progression and pancreatic pathology showing amyloid deposits as the dysmetabolic disease progresses [11].

Here we report on our standard method of performing an IVGTT in NHPs as part of our colony characterization of metabolic status in these animals. This method is easy to perform relative to other, more time consuming and costly techniques [2]. The IVGTT is useful for characterizing a large colony of animals rapidly and frequently. When taken into consideration with the level of glycated hemoglobin (HbA1C), the animal's diet and food intake history, as well as their percent lean mass and body fat, the IVGTT is normally sufficient for characterizing an animal's metabolic status and progression toward overt diabetes [6, 8].

HbA1C represents the average glycemic level over the life of a red blood cell, providing a reliable measure of glucose levels over the previous six weeks to three months. When measured from the fasted baseline blood sample of the IVGTT, this value provides a window into glycemic control during the months between procedures. If the animal has transitioned from dysmetabolic to diabetic since their last IVGTT, an HbA1C value much higher than their previous value would indicate that the transition began soon after their last IVGTT, whereas, an HbA1C value closer to their previous value would indicate that they have only recently transitioned. In general, in rhesus macaques, HbA1C values greater than 6% are considered abnormal, and indicate poor glycemic control [10, 23].

Glycemic levels should be interpreted within the context of the behavior and general health of the animal as a whole. Diabetic macaques — like humans — exhibit hyperphagia, polydipsia, and polyuria. Group housing of animals provides significant challenges to measurement of these indicators and the individual care required for dysmetabolic and diabetic monkeys. We recommend singly housing the animals in order that more personalized care may be provided, and behavioral markers of the health of the monkey more easily be monitored [8]. Additionally, diabetic macaques will exhibit weight loss, as well as an elevated lipid profile (increased cholesterol, hypertriglyceridemia) and disturbed mineral metabolism in serum chemistry. It is important to measure markers of liver and kidney function in serum chemistry, as damage to these organs are often complications of advancing metabolic disorder/diabetes, and may be co-determinants of glycemic, lipid and mineral imbalances [9, 11, 18, 24].

When using this method, the historic values generated from multiple, frequent characterizations over the life of a monkey are of particular value. If other procedures, such as a glucose clamp or graded glucose infusion (GGI), are needed to fully assess an animal's health, it is commonly upon initial characterization when their history is unavailable. However, once a baseline has been established, repeated IVGTTs of a frequency of every three months are normally sufficient to track an animal's progress. This is particularly important when the animals are enrolled into multiple studies throughout a calendar year based upon their metabolic status. While their health may remain relatively stable for years at a time, when the metabolic status of an animal worsens, a dramatic increase in insulin resistance and glucose intolerance can occur very rapidly. HbA1C values allow for some interpolation of the decline or improvement of the health status of the animal between procedures scheduled three months apart. For this reason, this method is ideal for characterizing animals used in multiple, longitudinal studies over the course of their natural lifespan.

Protocol

All animal procedures were approved by the David H. Murdock Research Institute IACUC located on the North Carolina Research Campus (NCRC), under protocol 14-017, Characterization of a nonhuman primate model of diabetes and prediabetes/insulin resistance and efficacy of therapeutics to improve insulin sensitivity and metabolic function.

1. Animal Selection and Study Preparation

1. Select diet and weight stable animals based on monthly food intake and body weight records.
   NOTE: Animals that have exhibited a recent decline in appetite should not be characterized until their food intake has stabilized.
   1. For mature animals (> 5 - 6 yr), do not select animals whose body weights between consecutive months differ by more than 10% without first examining the monkey and ruling out causes other than changing metabolic status for the dramatic change in weight.

2. For glucose, insulin and c-peptide analytes, prepare K_2EDTA sample collection tubes with protease inhibitor (Aprotinin and DPP4I).
   NOTE: The DPP4I + Aprotinin cocktail provides a broad spectrum of protease inhibition should this be required for additional analytes (glucagon, GLP-1). In the event that additional analytes are not collected, the blood tubes should be prepared in the same way to maintain consistency in sample collection. Samples for assays not validated for this method should be collected separately, according to the recommendations of the manufacturer.
   1. Prepare the protease inhibitor cocktail by mixing 100 mg lyophilized Aprotinin with 10 ml of DPP4I. Add 10 µl of the Aprotinin + DPP4I mixture to each blood tube for every milliliter of blood collected.
   2. Add an additional 10 µl of the protease inhibitor cocktail to each tube for possible collection overage. Store the treated-blood tubes at -20 °C until use. Keep the tubes on wet ice during the procedure and spin at 4 °C.
   3. Use a serum separator tube to collect a blood sample at baseline for standard serum chemistry analysis. For the Complete Blood Cell count (CBC), use a standard K_2EDTA without protease inhibitors. Use cryovials to aliquot plasma and serum after the blood samples have been spun down.
   4. Label the blood tubes and the cryovials appropriately with the animal identification, date, procedure, timepoint, and sample volume. Label the cryovials with the analyte(s) in plasma for appropriate assay.

3. Prepare the heparinized saline flush by injecting 0.15 ml of 1,000 USP units per ml heparin into a 250 ml bag of normal saline. Obtain a solution of 0.06 mg heparin/ml. Draw 40 - 60 ml of this solution into a saline lock for flushing between samples. Draw an additional 1 ml and 5 ml into separate syringes for flushing the dextrose infusion port before and after the infusion, respectively.

2. Animal Sedation and Preparation

1. Remove food from the animal's cage no less than 14 hr before the procedure, and no more than 18 hr.
   NOTE: It is important that animals be fasted for the procedure to avoid any post-prandial variation in glycemic values. It is also a precaution to avoid regurgitation and aspiration of stomach contents while anesthetized.
2. Sedate animals for the duration of the IVGTT procedure with ketamine given intramuscularly as a general anesthetic, at 10 mg/kg. Administer additional ketamine (5 - 10 mg/kg) at intervals of 20 - 30 min, or as needed, during the procedure.
   1. Weigh the sedated animal. Place the animal in a laterally recumbent position on a heated procedure table.
   2. Monitor clinical parameters every 15 to 20 min to ensure the animal is in a stable plane of anesthesia. Measure heart rate (100 - 200 bpm) and SPO_2 (> 92%) with a pulse oximeter. Measure respiratory rate (20 - 50 breaths/min) with a stopwatch, counting respirations visually or by hand over fifteen seconds and multiplying by four. Measure temperature (> 97 °F) rectally. Monitor the color of the mucous membrane around the gum and lips (moist, pink).

3. Prepare two cannula sites. Use hair clippers to trim the hair from the area of interest where the catheter will be inserted, and sterilize the entire region with alternating scrubs of chlorhexidine and 70% alcohol.
   1. Place one catheter in the region of the left or right cephalic or saphenous veins and attach it to a heparin saline flush (0.06 mg heparin/ml) with a three-way stopcock. This is the sampling blood draw site.
   2. Place the second catheter in another leg or arm in the region of the cephalic or saphenous veins and attach a port. Use this site for the dextrose infusion. Use a small, 1 ml flush of heparinized saline to keep the cannula patent prior to dextrose infusion.

3. IVGTT Procedure

NOTE: The IVGTT procedure consists of 8 blood draw sampling time-points (**Table 1**).

1. Take the baseline sample and use a hand held glucometer to measure the fasting blood glucose level. Obtain a serum sample for standard chemistry analysis, as well as a whole blood sample for a CBC in order to assess the overall health of the animal. Collect plasma samples to assay glucose and insulin levels from the baseline sample using a kit validated for use with macaques as per the manufacturer's instructions [8, 9].
   NOTE: It is important to have a pre-draw of 0.5 ml taken from the cannula prior to taking any sample for blood collection to remove residual blood or heparin in the dead-space of the cannula.
2. After obtaining the baseline sample, infuse the dose of 50% dextrose (250 mg/kg) over 30 sec into the dextrose infusion port.
   NOTE: Higher dose models (500 mg/kg) can be used, though the dose should be fixed across procedures in order to make longitudinal comparisons.
   1. Flush the infusion port with 5 ml of heparinized saline to make sure that there is no dextrose left in the port. The end of the infusion is the T0. Have the technician replace the gloves, as residual dextrose from the infusion may contaminate subsequent blood samples.
3. The first post-infusion sample time point is at T3 min, from the end of dextrose infusion, followed by T5 min, T7 min, T10 min, T15 min, T20 min, and the last sample time point is at T30 min. Collect plasma from each timepoint to assay glucose and insulin levels with the baseline sample (see step 3.1).
   1. At the T3 min time point, use the hand held glucometer to once again check the blood glucose level.
      NOTE: The glucometer readings at baseline and T3 are only to confirm the infusion of the dextrose. The blood glucose level at the T3 min time point should be ~100 mg/dl higher compared to the fasting baseline plasma glucose level.

4. Animal Recovery and Sample Processing

1. Remove the cannulas and apply pressure to the catheterized sites for hemostasis after the T30 min time-point. Monitor the animal until it has regained consciousness and is sitting up. Offer feed once the animal is fully recovered.
2. Immediately place each whole blood sample into K_2EDTA tubes on ice. Centrifuge at 3,000 rpm at a temperature of 4 °C within 10 min of collection. Aliquot plasma samples into cryovials, freeze and store at -80 °C until analysis.
   1. Allow the blood into the serum tube for standard serum chemistry analysis to sit at room temperature for no less than 20 min and no more than 30 min before centrifugation at 3,000 rpm at RT. Freeze the serum samples until assayed within 48 hr of collection.
      NOTE: Refrigerate the whole blood samples collected for CBC analysis until assayed within 24 hr of collection.

5. Data Treatment

1. After establishing the plasma insulin and glucose curves, determine the **glucose clearance rate *K*** from the slope of the natural log of glucose values above baseline [16, 17].
   NOTE: A healthy NHP may be expected to have a **glucose clearance rate *K*** well above 1, often greater than 2 or more, as a healthy animal will often return to their baseline glucose values within 30 min. As insulin production drops off, the glucose clearance rate K will drop more dramatically, falling below 1.
2. Calculate the **AUC** as a whole, as the sum of totals of the area of the trapezoids representing the area under the curve of each line segment between time-points, through T30 [16, 17].
   NOTE: Traditionally, the **AUC** of the first ten minutes of the procedure is considered the acute insulin response to glucose (**AIR**), while the **AUC** from the last 20 min of the procedure is considered the late insulin response (**LIR**). As the animal becomes more dysmetabolic, the insulin **AUC** will increase, reflecting compensation for increasing insulin insensitivity. As the animal transitions to overt diabetes, however, the **AUC** will decrease, often initially in the acute insulin phase, captured by the **AIR**.

Representative Results

The results shown in **Figure 1** are demonstrative of typical glucose and insulin curves from mature, healthy and diabetic cynomolgus macaques over the course of a 30 min IVGTT. Data from healthy and advanced diabetic monkeys are shown in order to contrast the obvious differences between animals from both extreme ends of the range of metabolic characterization. This IVGTT protocol has been used successfully by the authors in rhesus macaques with similar results.

The fasted, baseline glucose values of a healthy macaques (rhesus and cynomolgus) can be as low as 50 to 60 mg/dl [8]. As illustrated in the figure, the initial glucose excursion — measured at T3 — for a healthy NHP may not climb as high as a typical baseline glucose value for a diabetic animal, which is frequently in excess of 200 mg/dl. Over the course of the following thirty minutes, the glucose levels of a healthy animal often return to their baseline values, while dysmetabolic and diabetic animals do not (**Figure 1**; solid lines). The accompanying insulin curve for a healthy animal exhibits two peaks (**Figure 1**; dashed blue line), which correspond to the initial, rapid decline in blood glucose (phase 1) mediated in larger-than-normal part by peripheral effects of insulin on glucose transport and uptake [3, 4]. The magnitude of these peripheral effects, even during the first phase of the insulin response, does not equal the contribution of the liver to glucose lowering, which, during the smaller but more sustained second insulin peak (phase 2), has the greatest impact on glycemic levels via suppression of endogenous glucose production[1].

As an animal progresses from healthy to dysmetabolic, there is typically a diminution of the first phase of the insulin response to the dextrose bolus, which may be reflected in a reduced **AIR**. However, the **AUC** may remain unchanged, as there will more frequently be an overall increase in insulin production, providing for largely unchanged glycemic levels over the course of the procedure as the increased insulin production compensates for the decrease in sensitivity. Once an animal has become overtly diabetic, insulin production drops dramatically in response to the dextrose bolus (**Figure 1**; dashed red line). Glycemic levels will remain elevated over the course of the procedure, as what glucose clearance occurs is now mediated almost entirely by non-insulin-dependent mechanisms (**Figure 1**).

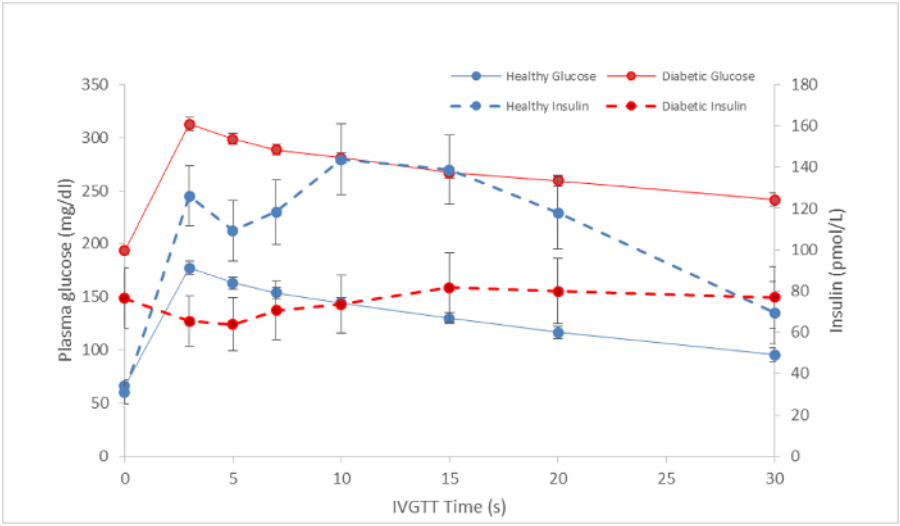

Figure 1: IVGTT Glucose Clearance and Insulin Production Curves for Diabetic and Healthy Control Animals. Shown here are glucose (solid line) and insulin (dashed line) curves for healthy (blue line) and diabetic (red line) animals. These values are averages of actual data collected during IVGTTs performed on cynomolgus macaques (diabetic glucose: n = 27; healthy glucose: n = 21; diabetic insulin: n = 23; healthy insulin: n = 20; standard error bars shown). Please click here to view a larger version of this figure.

Clock Time	Time points (min)	Real Time	Blood Sample amount (ml)	Sample Assay	Glucose Meter Reading (mg/dl)	
	Baseline		5 ml	**1.5 ml SST** - chemistry **0.5 ml K_2EDTA** - CBC, HbA1c **3 ml K_2EDTA + 50 µl prot.** - glucose, insulin, C-Pep		
	0		Dextrose infusion 250 mg/kg IV, given over 30 sec, followed by 5 ml Heparin saline flush			
	3		2.0 ml	K_2EDTA + 30 µl Prot.: Glucose, Ins, C-Pep		
	5		2.0 ml	K_2EDTA + 30 µl Prot.: Glucose, Ins, C-Pep		
	7		2.0 ml	K_2EDTA + 30 µl Prot.: Glucose, Ins, C-Pep		
	10		2.0 ml	K_2EDTA + 30 µl Prot.: Glucose, Ins, C-Pep		
	15		2.0 ml	K_2EDTA + 30 µl Prot.: Glucose, Ins, C-Pep		
	20		2.0 ml	K_2EDTA + 30 µl Prot.: Glucose, Ins, C-Pep		
	30		2.0 ml	K_2EDTA + 30 µl Prot.: Glucose, Ins, C-Pep		

Table 1: IVGTT Procedure Table. Shown here is a standard procedure report for an IVGTT, detailing all the pertinent information captured during the procedure. Clock times for sample draws following the dextrose infusion should be calculated from the end of the infusion. Actual times should be recorded for each draw. Please click here to download this table as a Microsoft Word document.

Discussion

The IVGTT assesses the capacity of glucose-stimulated insulin release by a single dextrose infusion based on body weight [5, 12, 13]. From the assay, the fasting blood glucose and insulin level is attained, and it allows an assessment of the animal's capacity to release insulin and return the elevated glucose level towards baseline. This provides the user with information to characterize the animal as a normal glucose and insulin level healthy control, a hyperinsulinemic dysmetabolic animal with normoglycemia, or a hyperglycemic insulin-resistant diabetic animal.

It is important that blood samples, particularly from the early time points, after infusion of dextrose, are drawn and processed in a timely and consistent manner. This will reduce variability within and between subjects, and decrease opportunities for errors in the temporal order of the data. The timing of the T3 is of critical importance because the excursion of plasma glucose from baseline to three minutes after infusion of the dextrose is one of the endpoints that is used to characterize the metabolic status of the animals. There is a decline in glucose clearance within the first 7 - 10 min after the dextrose infusion, which may be missed if those blood draws are not taken on time. It is the area under this portion of both the glucose and the insulin curves that is used as an endpoint, emphasizing the importance of these early time points. If a blood draw after the T3 is taken late, the procedure can continue, but the actual time of the draw should be reported in order to preserve the true shape of the curve as much as possible. It is particularly important that deviations of more than a minute be captured and reported. After the first ten minutes, glucose clearance tends to become more gradual, and may not return to baseline within 30 min (**Figure 1**). The procedure can be extended by sampling every 10 min after the T30 to capture how long it takes the animal to return to baseline.

IVGTTs are limited by their ability to directly measure insulin sensitivity. It is primarily useful as a method for assessing insulin production and glucose clearance. However, hepatic insulin extraction from the portal blood supply compromises measurements of "insulin secretion" from the systemic blood supply. This can be partially overcome, however, by measuring c-peptide, which is secreted in equimolar amounts from the pancreas but is not removed by the liver before entering the general blood supply. This gives a clearer view of insulin production to better assess β-cell function [16]. Also, IVGTTs do not distinguish between insulin-dependent mechanisms of glucose clearance and non-insulin dependent mechanisms [1, 6]. While insulin sensitivity may be assessed indirectly through minimal modeling of data from an IVGTT, more reliable measures can be achieved through the use of procedures such as the Graded Glucose Infusion (GGI), which provides a β-cell dose-response curve [14, 15].

However, insulin sensitivity is still assessed only indirectly with the GGI. A standard insulin dose can be administered to the animal during the IVGTT to improve estimation of insulin sensitivity, but that will mask endogenous insulin production, compromising assessment of β-cell function. The Hyperglycemic and the Hyperinsulinemic Clamp techniques measure directly glucose regulation via controlled insulin exposure. The drawback to both the GGI and the Clamp techniques is that they are expensive to perform and require more personnel and time to complete (sometimes as long as six to eight hr, depending on the design of the procedures) [16]. The IVGTT, on the other hand, can be performed under an hour and with minimal manpower.

Generally, a healthy animal will dispose of glucose very rapidly, often returning to their baseline values within 30 min, and their insulin curve will exhibit two distinct peaks for the acute and late insulin responses. As an animal becomes more dysmetabolic, their fasted glucose and their glucose excursion in the first three minutes after infusion may increase. However, these values may vary widely among healthy animals, and are most informative of disease progression when compared to historical values from the same animal. Overall, however, the glucose clearance of a dysmetabolic animal may not differ greatly from when they were healthy. The first signs of disease progression are going to be most evident in the insulin curve. While the fasted insulin level may only be mildly elevated, the insulin **AUC** will typically increase dramatically in dysmetabolic animals, reflecting compensation for the developing insulin insensitivity. As β-cell function declines, though, a blunting of the acute phase insulin response may be seen. This will be reflected in a decreased **AIR**, which, when normalized to baseline, may approach 0 in a diabetic monkey. This is followed by an overall decline in insulin production and a dramatic decrease in glucose clearance [8, 9, 10, 11].

The metabolic status of an animal may be hard to determine with a single IVGTT. This can be the case when trying to distinguish an animal as either early dysmetabolic or pre-diabetic. When becoming progressively more dysmetabolic, insulin production increases to maintain euglycemia. While fasted glucose values may become slightly elevated, impaired glucose disposal does not become very evident until β-cell function has been compromised and insulin production begins to decline [18, 20]. For a period of time, before overt diabetes has set in, insulin values may resemble the curve of an early dysmetabolic animal in whom the first phase response has been compromised, but some insulin is still being produced above fasted, baseline values. Because of this, the AUC of the insulin curve of an early dysmetabolic and prediabetic animal can be very similar. In this situation, an elevated glucose curve, being impacted by both insulin-dependent and non-insulin-dependent mechanisms, is not sufficient to distinguish the animal as either early dysmetabolic or pre-diabetic. This distinction may be made by performing a hyperinsulinemic/euglycemic clamp, which will directly demonstrate insulin sensitivity [19]. Alternatively, this distinction may be made upon performing another IVGTT on the animal after several months. In the latter case, if the AUC of the insulin curve increases, the animal may be considered to have been early dysmetabolic at the time of the first procedure. If the AUC diminishes, however, the animal's dysmetabolic status may be considered to have been advanced/prediabetic at the time of the first characterization. A prediabetic state may also be accompanied by weight loss and increased fluid intake during the period of time intervening the two IVGTT procedures. These circumstances illustrate why the IVGTT is a particularly useful tool when being considered in light of a history of such procedures as opposed to being used as a snapshot of an animal's health.

The use of chair-restraint with conscious animals has been demonstrated to produce a significant elevation in blood glucose levels during glucose tolerance testing [25]. For this reason, animals are sedated for this procedure. However, care must be exercised in the choice of anesthetic. It has been reported that the use ofα2-adrenoceptor agonists such as xylazine and dexmedetomidine for sedation can significantly increase blood glucose levels in nonhuman primates [17, 21]. The method reported here only uses ketamine for sedation, which does not cause blood glucose levels to rise [22]. On occasion, an animal may exhibit such rigidity and tensing that the technician may feel a relaxant is necessary. In such cases, Diazepam and Telazol have been demonstrated to have a much less profound effect on insulin production and glucose metabolism then α2-adrenoceptor agonists [21]. Hence it is important to consider the anesthetic regimen in metabolic tests, such as the IVGTT. In summary, the IVGTT is a simple metabolic function test that is routinely performed in NHPs, yet it can provide valuable information to categorize

animals within a colony into different metabolic states, hence informing their animal care and health management, as well as their potential utility in models of metabolic disease.

Disclosures

The authors are affiliated with a contract research organization (Crown Bioscience) active in the field of metabolic disease.

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