Lost in Translation

David H. Wasserman, Julio E. Ayala, and Owen P. McGuinness

There are few methodologies that have done more to advance our understanding of insulin action in humans and animals than the hyperinsulinemic-euglycemic clamp (henceforth referred to as the insulin clamp or clamp). The clamp was first developed for use in humans by DeFronzo, Tobin, and Andres at Johns Hopkins in 1979 (1). This technique was transferred to the rat in 1983 (2,3) and the mouse in the early 1990s (4,5). The insulin clamp is often combined with isotopic methods so that the investigator cannot only determine a total whole-body insulin action but also how specific tissues and metabolic pathways are affected. The adaptation of the insulin clamp for use in the mouse has been critical to the characterization of the still growing number of mouse models with modifications to the genes involved in the regulation of glucose and energy homeostasis. Despite the importance of this tool, a significant deficiency has evolved in the process of translating the clamp from humans to mice. The first articles reporting insulin clamps in the mouse conscientiously presented methods and results (4,5). However, since these first studies in the mouse, the care and conventions of the human clamp literature that immediately followed its development have largely been traded for no, minimal, or inaccurate description of methods and inadequate presentation of results. The reader is provided with too little information to independently interpret the results of an article. Instead, the reader is solely dependent on the conclusion to which he or she is directed by the authors. The perception that standards in the human and rat literature have lowered in recent years was brought to our attention during the preparation of this article. Here, we focus on the mouse literature. Of course, sound reporting of clamp methods and results applies to all species. The purpose of this article is to see how the careful presentations of the initial human insulin clamp studies might be better translated to the mouse insulin clamp literature. We will outline the problem and cite specific examples where failure to present clamps in a complete and accurate manner has had a negative impact. We will convey our view as to what investigators using the insulin clamp should include in published reports and what reviewers should require of them so that the reader can independently interpret the experiment.

Vagaries. The insulin clamp is challenging and subject to variations that are specific to the laboratory in which they are conducted. Despite these challenges and nuances, the clamp has been the object of very little scrutiny when applied to the mouse. Ayala et al. (6) first reported this deficit in 2006. Our sense is that some improvements have occurred with regards to the description of insulin clamps since this first statement of the problem. Nevertheless, many problems remain. Let us provide some examples. In 2004 and 2005, nearly half (44%) of studies in mice failed to report a sampling site (6). In the 2 years following the publication of Ayala et al. (6), this number has dropped to ~30%. Although this is an improvement, failure to indicate a sampling site in nearly one-third of published studies remains a deficiency in the field. One area where little has changed in the mouse clamp literature is the reporting of insulin doses and insulin levels. Prior to 2006, 48% of studies in mice mention a priming dose of insulin but fail to indicate the actual dose. For reasons described in detail later, this is not a trivial point. Furthermore, in almost all of these studies, absolute insulin levels (fasting and clamp) were not reported. It is impossible for a reader to know that hyperinsulinemia has been achieved and insulin levels are comparable between groups if insulin levels are not reported. Since 2006, the percentage of published reports that fail to indicate the priming dose of insulin and insulin levels was still 40%. Finally, to date ~90% of reports where insulin clamps were performed in mice do not show a time course of glucose levels and glucose infusion rates. Without this information, one cannot know whether a steady-state clamp has been achieved. Nearly all clamp experiments in humans present these three parameters: sampling site, insulin dosing/levels, or time course of glucose and glucose infusion. It is therefore unclear to us why standards for the mouse clamp literature should be any different.

The previous paragraph describes some fundamental omissions in the mouse clamp literature. Without the presentation of fundamental clamp components such as time course of glucose and glucose infusion it is impossible to know whether a clamp has been performed. This is not to say that from time to time certain vital information may be inadvertently left on the cutting room floor. A minimalist approach to the description of procedure and presentation of results is almost convention. A curious tangential observation is that whereas insulin clamps in the mouse are often poorly described or not described at all, glucose tolerance tests and insulin tolerance tests are described reasonably well. Methodology is usually adequately presented, and time courses are shown. The results from well-conducted glucose tolerance tests and insulin tolerance tests are more interpretable than an insulin clamp that does not report clamp insulin concentrations. In the same vein, there are articles that provide little to no information on the clamp but will describe a routine published buffer system or assay with great detail. The question thus remains, why has the insulin clamp been lost in translation to the mouse?

An example illustrating the vagaries that are pervasive in the mouse clamp literature is evident in the ambiguous

From the Department of Molecular Physiology and Biophysics, Vanderbilt Mouse Metabolic Phenotyping Center, Vanderbilt University School of Medicine, Nashville, Tennessee.
Corresponding author: David H. Wasserman, david.wasserman@vanderbilt.edu.
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descriptions of how blood samples are obtained. One common method during a clamp for obtaining blood from a mouse is to bleed it by slicing the tip of the tail off and compressing the tail so that blood drips from it (6). Some choose to restrain the mouse during this procedure. The advantage of this procedure is that it does not require the technically difficult implantation of an arterial sampling catheter, which is another common method for obtaining blood samples (6,7). The drawback is that blood does not always flow freely from the cut tail necessitating constant squeezing of the tail, especially when larger samples are needed for hormone and isotopic tracer analysis.

Except for Ayala et al. (6), no published report has indicated levels of stress hormones when using cut-tail sampling during insulin clamps in mice. Indeed, several otherwise nonideal approaches that have been taken might minimize the perception of stress in a study. These include *1*) glycogen depletion via overnight fasting that limits stress-induced hyperglycemia, *2*) beginning clamps with a pharmacological bolus of insulin that lower stress-induced hyperglycemia, and *3*) presentation of average, rather than time course, glucose levels that dampen the appearance of glucose variability induced by stress. Another commonly used approach is to state that a "tail-restraint" method was used to conduct experiments with "minimal stress" (8). Minimal stress is not defined, and there is no indication that this was evaluated with a scientific method. Furthermore, the description of the sampling method in this way is deceptive because it does not indicate that *1*) the tip of the tail has been cut off and *2*) the entire mouse, and not just the tail, is restrained. Furthermore, it does not describe the means by which the tail is compressed to obtain blood from the wound. This is significant because this means of handling the tail is the primary source of stress that occurs due to this sampling method (6). More recently, a statement to the effect that blood was obtained from tail vessels (9) has also been used as a concise way of indicating that the tip of the tail was cut, the mouse was restrained, and blood was dripped from the wound. The shortcoming with all of these common approaches to describing the insulin clamp is that while they reduce the perception of stress by the person reading the manuscript they have no bearing on whether the mice are stressed. The approach of cutting the tail to obtain blood has been used in our laboratory (10) and is of value for mice that do not handle the carotid catheterization surgery well (e.g., *ob/ob* mice). In addition, the cut-tail approach is a valuable tool because the surgical skills required for carotid catheterization are quite specialized. Useful information can certainly be and has been obtained from the approach of obtaining blood from the cut tail. However, it is important to be transparent about the sampling technique used in an experiment because the results may differ due to stress or other factors.

The use of a carotid artery catheter for sampling is not without problems (i.e., difficult surgery, longer postoperative period, and possibility of stroke). Different sampling methods have advantages and disadvantages. The important distinction is that investigators who use a carotid artery catheter in experiments are clear in the reporting, whereas many investigators who use the tail are not. Regardless, the need for accurate reporting is not trivial because differences in stress induced by sampling method may exist (6) and also the composition of the sample is different because the blood dripped from the tail is arterial and venous (plus a small amount of lymph).

One factor that has allowed for the vagaries of the mouse clamp to evolve is the illusion that a seminal reference exists that contains all the missing information. In a few cases, appropriate clamp method references do exist (6,7,11,12). Often the reader will be sent on a trail of unrelated citations. Methods reporting of an insulin clamp should at the very least include *1*) surgical procedures if applicable, *2*) infusion and sampling ports, *3*) insulin delivery protocol, *4*) isotope delivery protocol if applicable, *5*) fast duration, *6*) restraint or anesthesia if applicable, and *7*) sample frequency for clamp feedback measurements of blood glucose.

Reductionism. There are some very difficult issues associated with performing clamps on the conscious mouse. One such limitation pertains to the relatively small volume of blood in the mouse (~2 ml). Clearly, one has to scale down assay volumes and be judicious in sampling. Circulating glucose must be measured at frequent intervals throughout the clamp. However, measurements of clamp insulin and isotope concentrations usually must be limited to the interval just prior to the end of the clamp when insulin action is in a steady state. Similarly, sampling for the measurement of stress hormones usually must be limited to the end of the clamp. Erythrocyte replacement can sustain hematocrit and allow for more blood sampling. The small blood volume of the mouse does not allow for the same scope of blood sampling that can be undertaken in the human and large animal models. Sampling volume can be increased by infusing blood from an appropriately matched donor mouse. This, however, adds some complexity to the experiment and requires an increase in the mouse colony size. Clearly, the limitation in blood volume is all the more reason to present the data that are obtained during a clamp.

Blood or plasma glucose concentrations are measured throughout an insulin clamp. The measurement of circulating glucose online is the feedback from which the glucose infusion rate used to maintain euglycemia is based. A study is not a clamp without the regular measurement of glucose and the iterative adjustment of glucose infusion. Even though these data must exist if a clamp was truly performed, they are rarely presented. These are vital data because they demonstrate the quality of the clamp, whether steady states in blood glucose and insulin action were obtained, and the kinetics of insulin action (i.e., the rapidity of onset). These data are not at all trivial, especially when one considers that the rate of glucose flux is approximately seven times higher in mice than humans. This means that for a given mismatch in glucose infusion rate to glucose requirement, a greater deviation in circulating glucose will occur in mice than humans. As noted above, almost every insulin clamp study conducted in humans shows time courses for blood glucose and glucose infusion rate. Despite the added vulnerability of mice to deviations in blood glucose, very few insulin clamp studies conducted in mice show a time course of glucose and glucose infusion rates. Instead, the convention in the mouse clamp literature is to present average values for these parameters. Thus, the content of a clamp that generally lasts about 2 h is reduced to a single point. One is left to speculate in many cases what that single point represents. The consequence of this reductionism is that it is impossible for any reader to interpret whether a clamp was truly achieved.

Results from an insulin clamp are meaningless if the absolute fasting and clamp insulin levels are not reported.

How else would a reader know whether comparable hyperinsulinemia had been achieved? Differences in insulin concentrations can occur between genotypes, in response to dietary manipulations, or due to drug treatments, even when the insulin infusion rate is identical (13–15). Given the importance of this parameter, it is perplexing that many investigators performing clamps on mice do not report insulin concentrations. Instead, the convention has been to state that insulin infusions were done to "achieve physiological hyperinsulinemia." Actual empirically determined insulin measurements are not, however, reported. The use of speculated plasma insulin concentrations is problematic on many fronts. As mentioned, experimental manipulations can affect insulin levels among groups receiving equal insulin infusions. Furthermore, this practice renders the definition of "physiological hyperinsulinemia" as arbitrary. Illustrating this, speculated insulin concentrations obtained with an insulin infusion rate of 15 $pmol \cdot kg^{-1} \cdot min^{-1}$ from one laboratory fell from 850 (16) to 780 (17) to 650 (18) to 600 (19) to 400–500 (20) to 350 (21) to 300 (11) to 200 (22) pmol/l over a period ranging from 2000–2005. These declining estimates were made independent of genotype or treatment and apparently independent of actual chemical analysis.

Results reporting an insulin clamp should at the very least include *1*) the time course for circulating glucose and the glucose infusion rate, *2*) an index that shows whether or not glucose-specific activity is constant during the steady-state sampling period if applicable, *3*) plasma insulin concentration before and during the clamp, and *4*) hematocrit. If possible, an end-study stress hormone level or some other stress marker would provide a useful validation test of the clamp procedure.

Perpetuity. Up to this point we have outlined deficits in the presentation of the methods and data. Here, we show why this is a very real problem. Like any other experiment, the study design determines the outcome of an insulin clamp in mice (6). Although certain modifications have improved the methodology, other variations have arisen in insulin clamp protocols that appear to be based on conceptual flaws. Two such modifications, as discussed below, have been incorporated into a significant component of the literature and are still being used by some laboratories. These conceptual errors have persisted in the literature because the descriptions of the pertinent protocols are generally inadequate to identify the flawed procedure. Thus, the failure to provide methods in articles has had repercussions. The absence of peer review of these flawed techniques has allowed them to infect the literature in perpetuity. These major flaws were eventually uncovered when the methodology was finally described in a careful and accurate manner (23). The unfortunate reality that became clear is that approximately half the literature up to that point appears to carry these significant conceptual errors. These errors are described below.

One mistake is that the insulin priming dose used in many studies is 50% of the total insulin administered and ~20 times the amount needed to expedite steady-state insulin concentrations. The origin of this mistake can be traced from information in the study of Haluzik, Gavrilova, and LeRoith (23), and it is likely contained but not described in more than 50 published reports. A study of the impact of the large insulin bolus has been performed in wild-type mice (6). Complicating the impact of the insulin megabolus is the probability that the resulting supraphar-macological insulin concentration will be dependent on the model being studied (i.e., genotype or pharmacological treatment). First-phase insulin secretion has persistent effects that last well after its cessation (24,25). One can equate the large insulin bolus to a hyperexaggerated first-phase insulin secretory response. The use of an excessively large insulin bolus is still common, although in some laboratories it has been scaled down to ~10 times the amount calculated to expedite steady-state insulin concentrations (15,26,27).

The second modification to the standard insulin clamp that crept into protocols used in some laboratories is that a bolus of [3-^{3}H]glucose (10 μCi) is given in the middle of the study. This priming dose is administered after a [3-^{3}H]glucose steady state has already been obtained! This is in addition to or in place of the correct [3-^{3}H]glucose priming dose that is administered at the start of the tracer infusion. The incorrect tracer prime is administered so that it coincides with the onset of the primed insulin infusion. This makes sense qualitatively if one assumes that the insulin dose increases the glucose volume of distribution (i.e., accumulation of intracellular glucose). The problem is with the magnitude of the tracer bolus. A tracer primer is calculated as the product of the tracee (glucose) pool size and the target glucose–specific activity. Under commonly used insulin clamp conditions, it could be projected that a bolus of 10 μCi is large enough to label the glucose pool if it filled the entire body-water space at concentrations comparable with blood glucose (assuming a [3-^{3}H]glucose infusion rate of 0.1 μCi/min). The large tracer bolus disrupts blood [3-^{3}H]glucose steady state and floods the system with radioactive glucose. The excess [3-^{3}H]glucose should be quickly cleared from the blood. The problem is that glycogen and H_2O pools become labeled with ^{3}H. If one begins with information provided by Haluzik, Gavrilova, and LeRoith (23), it is possible to roughly estimate the number of articles that are affected. Like the insulin megabolus, the excessive ill-timed tracer bolus is probably also contained but not described in more than 50 articles.

For the same reason these problems arose (poor reporting of methods and results), it is impossible to know all the articles that are marred by the technical and conceptual mistakes outlined above, short of investigators submitting corrigenda. It is also difficult to know whether other conceptual problems have infected the literature. Looking forward, the solution to this problem is for reviewers and editors to require transparency in the description of methodology and presentation of results.

SUMMARY

Performing an insulin clamp is not akin to performing an established chemical assay, in which all the reagents are well established. Yet based on the lack of technical descriptions and reductionism of results, one might assume that an insulin clamp can be done with a few good reagents and a kit. It should be clear that there are many acceptable ways to perform a clamp. The specific insulin clamp technique should accommodate the specific question being addressed as well as the technical strengths of the laboratory. Because there is more than one way to perform a clamp on a mouse, the particular clamp protocol that is used should be described accurately. As exemplified by the circumstances described above, it is in the best interests of the scientific community to provide an accurate description of the clamp method so that critical

mistakes may be identified. Two mistakes are described above. To some extent, these methods were corrected once they were finally identified (23). With regard to presentation of results, there is no evidence that a clamp was performed without presenting the time courses for circulating glucose and glucose infusion rates. Likewise, although it may seem obvious to measure insulin during an insulin clamp, numerous articles fail to do so, rendering such studies not interpretable.

One common refrain is that journals do not permit the space for description of methods and presentation of clamp results. There are other more reasonable ways to save space. Certainly, the uninformative presentation of blood glucose and glucose infusion rates as a histogram is wasted space that would better serve the reader if it were used to show a time course graph. Furthermore, the growing popularity of supplemental sections negates the argument that space is a limiting issue. Because the mouse is so difficult to study, it is logical and necessary to present the few indexes that are available.

Once presentation of methodology and results becomes routine, a serious and broader discussion of optimal clamp techniques can begin. The insulin clamp procedure used at the Vanderbilt Mouse Metabolic Phenotyping Center is posted in great detail at the center Web site (www.mc.vanderbilt.edu/mmpc). We realize that translation of the clamp method from humans to mice is still an evolving process. We have benefited in the past from comments and suggestions from participants in a course we offer each year on glucose clamping as well as from other investigators interested in clamp technology. We hope for a continued exchange of information and for experimental transparency that will improve the ability to glucose clamp the conscious mouse.

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