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Acclimation prior to an intraperitoneal insulin tolerance test mitigates stress-induced hyperglycemia in conscious mice.

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TITLE:

Acclimation Prior to an Intraperitoneal Insulin Tolerance Test to Mitigate Stress-Induced Hyperglycemia in Conscious Mice

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KEYWORDS:

Stress, hyperglycemia, insulin tolerance test, insulin action, mice.

SUMMARY:

Stress-induced elevations in glucose levels can confound interpretation of data derived from a conscious intraperitoneal insulin tolerance test in mice. In this article, we describe a method to acclimate the mice to handling, injections and blood sampling prior to performing the insulin tolerance test in order to limit stress-induced hyperglycemia.

ABSTRACT:

The insulin tolerance test is commonly used in metabolic studies to assess whole body insulin sensitivity in rodents. It is a relatively simple test that involves measurement of blood glucose levels over time following a single intraperitoneal injection of insulin. Given that it is performed in the conscious state and blood is often collected via a tail snip, it has the potential to elicit a stress response from animals due to anxiety associated with handling and blood collection. As such, a stress-induced rise in blood glucose can occur, making it difficult to detect and interpret the primary endpoint measure, namely an insulin-mediated reduction in blood glucose. This has been seen in many mouse strains, and is quite common in diabetic db/db mice, where glucose levels can increase, rather than decrease, after insulin administration. Here, we describe a method of acclimating mice to handling, injections and blood sampling prior to performing the insulin tolerance test. We find that this lowers stress-induced hyperglycemia and results in data that more accurately reflects whole body insulin sensitivity.

INTRODUCTION:

Metabolic tests in rodents are routinely performed to assess various parameters that regulate glucose homeostasis[1]. The gold standard for assessing whole-body insulin action in vivo is the hyperinsulinemic-euglycemic clamp[2]. This test involves administration of insulin to raise circulating insulin levels while glucose is infused to maintain euglycemia. The glucose infusion rate required to maintain euglycemia is indicative of insulin action. While it is a powerful tool in metabolic research, the clamp technique in mice is technically challenging and labor intensive, and thus is not well suited as an initial screening tool in characterizing a metabolic phenotype. For these reasons, the simpler intraperitoneal insulin tolerance test (ITT) is often chosen.

The ITT is performed in the conscious state following a fasting period (typically 4-6 hours). A bolus of insulin is administered intraperitoneally, after which blood glucose is monitored over a timeframe that usually lasts 60 min. Blood glucose levels are expected to fall due to the ability of insulin to facilitate glucose uptake into insulin-sensitive tissues; the degree to which this occurs is indicative of whole-body insulin action. In some cases, it has been shown that glucose levels paradoxically increase, rather than decrease, after insulin administration. This phenomenon is likely attributed to a stress response. Handling, injections and blood sampling can all induce stress[3-5], resulting in activation of the hypothalamic-pituitary-adrenal axis (HPA) and the autonomic nervous system (ANS)[6-8]. It is well known that both the HPA and ANS contribute to increases in circulating glucose levels[9-11]. The presence of stress-induced hyperglycemia at the beginning of an ITT is problematic, as it interferes with the rate and magnitude of the fall in glucose upon insulin administration[12]. This can lead to erroneous conclusions regarding the presence of insulin resistance. Thus, to mitigate the confounding impact of stress on glucose levels during an ITT, we have developed a method of acclimating mice to handling, injections and blood sampling prior to performing the ITT.

PROTOCOL:

All methods described here have been approved by the VA Puget Sound Health Care System's Institutional Animal Care and Use Committee.

NOTE: Local requirements for monitoring and/or intervention of animals that may experience hypoglycemia may differ from those described here.

1. Fasting (t= -210 min)

1.1. After the dark cycle has ended, transfer mice to a new cage with non-nutritious bedding such as cellulose or paper bedding (not corn-cob bedding, which will affect metabolic endpoints if consumed by mice[13]). Provide mice with ad libitum access to water throughout the fasting period. Be consistent with the fasting time of day and duration across groups of mice. For the data shown, remove food between 0700 and 0800 hours, which is 1-2 hours after the dark cycle has ended.

1.2. Move cage(s) to the location where the ITT will be performed. This should be a quiet space where stressors such as temperature, noise, light or movement are minimized.

2. Acclimation (t= -150 to -60 min)

IMPORTANT: Handle mice as gently as possible. Avoid use of a restraint device if possible.

2.1. At -150 min, measure body weight. This will be used for calculation of the volume of insulin that will be administered for the ITT.

2.2. Pick up the mouse gently by the tail and rest on a flat tabletop surface while still gently gripping the tail. Use a 20 G needle (or surgical scissors) to make a small incision in the tip of the tail. A drop of blood should begin to form at the site.

2.3. Set the mouse on a smooth hard surface and restrain the mouse gently by the tail.

2.3.1. To record blood glucose at this time, place a drop of blood from the tail tip on the test strip of a handheld glucometer. If necessary, **very gently** massage the tail to obtain a drop of blood.

2.4. Draw up 100 µL of sterile saline into an insulin syringe. Pick up the mouse using gentle scruffing and inject intraperitoneally. Record the time of saline injection.

2.4.1. If multiple mice are being studied, inject the mice with saline at 1 min intervals to allow time for subsequent steps below.

2.5. At 15 min after saline injection, pick up the mouse gently by the tail. Use gauze to gently dislodge any blood clot on the tail tip.

2.5.1. Optional, record blood glucose again at this time as described in step 2.3.

2.6. At 30 min after the saline injection gently pick up the mouse by the tail. If desired, obtain another blood glucose measurement as described in step 2.3.

2.7. Return the mouse to the cage, and repeat for other mice as necessary. Leave mice undisturbed until -90 min.

2.8. At -90 min, repeat steps 2.3 through 2.6, including measurement of blood glucose levels if desired.

2.9. Return the mouse to the cage, repeat for other mice as necessary. Leave mice undisturbed until the ITT (for which the baseline blood glucose measurement is done at -5 min).

3. Insulin Tolerance Test (t= -5 to +60 min)

3.1. Prepare a working solution of regular insulin (CAUTION) in sterile saline, such that the

desired dose can be injected at 4 µL/g body weight.

NOTE: Caution should be used when handling insulin as accidental injection can result in hypoglycemia.

3.2. Prepare a 25% (v/v) dextrose solution in sterile saline to have on hand in case mice develop hypoglycemia requiring intervention.

3.3. At -5 min, pick up the mouse gently by the tail. Determine the baseline blood glucose level at this time, by placing a drop of blood from the tail tip on the test strip of a handheld glucometer. If necessary, **very gently** massage the tail to obtain a drop of blood.

3.4. Draw up insulin working solution into an insulin syringe (4 µL/g body weight, for a typical dose range of 0.5-2.0 U/kg). Pick up the mouse using gentle scruffing and inject intraperitoneally. Record time of insulin injection.

3.4.1. If multiple mice are being studied, inject the mice with insulin at 1 min intervals to allow time for subsequent blood sampling and data recording below.

3.5. At 15 min after insulin injection, pick up the mouse gently by the tail. Use gauze to gently dislodge any blood clot on the tail tip. Determine the blood glucose again at this time as described in step 3.3.

3.6. Return the mouse to the cage and monitor for signs of hypoglycemia (e.g., excessive lethargy). If mice develop symptoms of hypoglycemia, measure blood glucose as described in step 5 and administer dextrose if necessary. A mouse undergoing dextrose intervention would be removed from the ITT protocol at this time.

3.7. Repeat steps 3.5 and 3.6 at 30 min after insulin injection.

3.8. Repeat steps 3.5 and 3.6 at 45 min after insulin injection.

3.9. Repeat steps 3.5 and 3.6 at 60 min after insulin injection.

3.10. Return the mice to their home cage, containing a few food pellets on the floor of the cage to aid in recovery from the ITT. Continue to monitor for 30 min and if mice have not regained normal activity/behavior follow procedure described in step 3.6.

NOTE: **Figure 1** summarizes the above acclimation and ITT procedure.

REPRESENTATIVE RESULTS:

Figure 2A and **Figure 3A** are representative data (individual and mean data, respectively) showing a paradoxical rise in blood glucose levels in diabetic db/db mice in the 15 minutes following insulin administration, consistent with stress-induced hyperglycemia. Note, no rise in

blood glucose was evident in the control non-diabetic littermates (db/+ or +/+) that underwent the same procedure. To determine whether acclimation to the ITT procedure was effective in mitigating this increase in blood glucose, the same mice underwent the acclimation protocol described above. Each major step is designated by vertical lines within the graph (**Figure 2B**) and blood samples were taken each time the mice were handled. Handling and/or saline injection at -150 min resulted in a rise in blood glucose in 3 of the 4 db/db mice (red circles); the fourth mouse had blood glucose levels >600 mg/dL. As 600 mg/dL is the upper limit of detection for the glucometer used, we were unable to ascertain whether blood glucose rose in the fourth mouse. The subsequent handling at -90 min resulted in increased blood glucose in 2 mice. Blood glucose levels were not affected by handling in control mice (blue triangles), though by t = 0, blood glucose levels were lower than at -60 min (150 ± 6 vs. 175 ± 8 mg/dL; n = 4, p = 0.02). Blood glucose levels during the ITT following acclimation decreased at all time points following insulin administration. Mean data for ITT before and after acclimation are shown in **Figure 3**. Note that overall glucose levels were lower in acclimated versus non-acclimated diabetic db/db mice (**Figure 3A**; $p < 0.05$). Further, the fall in blood glucose levels during the first 15 min after insulin administration was greater in non-diabetic littermate controls (**Figure 3B**; p = 0.002) following the acclimation protocol.

FIGURE AND TABLE LEGENDS:

Figure 1. Schematic depicting the handling, injection and blood sampling procedure during acclimation and the ITT.

Figure 2. Representative examples of ITTs performed with and without acclimation paradigm. (**A**) Blood glucose levels in diabetic db/db mice (purple circles) and littermate non-diabetic controls (db/+ or +/+; green triangles) during an ITT (2 U/kg) performed following a 3.5 h (210 minute) fast but no additional handling. (**B**) Blood glucose levels from the same mice (db/db in red circles, controls in blue triangles) subsequently undergoing the acclimation procedure described here, followed by the ITT (2 U/kg). The time interval between procedures in Panel A versus Panel B was 6 days. Data are shown for individual mice (n=4 per genotype).

Figure 3. Mean blood glucose data for ITT performed before and after acclimation
(**A**) Mean (± SD) blood glucose levels from the same db/db mice during ITT performed without (purple) or with (red) acclimation procedure; note that the acclimation procedure abolishes the rise in glucose levels between 0 and 15 min. Also, overall glucose levels are lower in acclimated versus non-acclimated diabetic db/db mice ($p<0.05$). n=4. (**B**) Mean (± SD) blood glucose levels from the same control db/+ or +/+ mice during ITT performed without (green) or with (blue) acclimation procedure; note that the acclimation procedure results in a steeper decline in glucose levels between 0 and 15 min (p=0.002). n=4.

DISCUSSION

The hyperinsulinemic-euglycemic clamp is considered the gold standard for assessing insulin action in vivo. Modifications to the methodology for performing the clamp have resulted in the technique being done in conscious, unrestrained mice[2] that have been previously catheterized using a two-catheter system[14] to enable blood sampling via the carotid artery and infusions via

the jugular vein. This limits the need to handle or restrain mice during the procedure, thereby reducing stress responses[2]. It is well recognized that stress can confound various endpoint measures in metabolic studies[1,3]. Stress can be induced by various factors in addition to handling, including blood sampling, olfactory stimuli, noise, light, and anesthesia[3-5]. Consequent activation of the hypothalamic-pituitary-adrenal axis (HPA) and the autonomic nervous system (ANS) can have profound effects on glucose levels[9-11].

While measurement of insulin action by clamping mice is the preferred method, it is often not feasible as it is technically challenging and labor intensive. For these reasons, the simpler ITT is more commonly performed in mice. ITTs monitor changes in blood glucose over time (typically 60 min) in response to an intraperitoneal bolus of insulin in mice that are fasted. A small volume of blood is sampled via tail tip at baseline and every 15 min thereafter. Considerations in performing ITTs have been previously discussed, particularly with respect to the period of fasting, the dose of insulin, and presentation of resulting data[1,12]. Here, we have considered the impact of stress prior to the ITT on the subsequent glucose response to insulin. We came to this from studies in db/db mice, where we consistently observed a paradoxical increase, rather than decrease in blood glucose following administration of insulin. Upon acclimating the mice to handling and injections prior to performing the ITT, we found that we could mitigate the rise in blood glucose. This suggests stress likely plays a role in the apparent hyperglycemia observed at the onset of the ITT. This phenomenon appears to be common in the literature. A PubMed search was performed using the search terms “db” and “insulin tolerance”, yielding 101 results (publication dates 1998 to 2019). Of those, 76 publications had accessible full text content, performed ITTs in db/db mice and displayed ITT data as glucose levels over time (raw values and/or percent of baseline values). Among those 76 studies, blood glucose levels were increased during the ITT in untreated db/db mice in 16 studies (21%). An additional 4 studies showed increases in glucose levels in other groups of mice (e.g., drug-treated db/db or control non-db mice). Finally, an additional 10 studies failed to observe a decrease in glucose levels following insulin administration in db/db mice over the course of the ITT, though this may have been related to the dose of insulin used or a state of marked insulin resistance in the mice. Thus, a greater awareness of the impact of stress is needed when performing ITTs, particularly in terms of how it may affect data interpretation.

To that end, we have developed an acclimation protocol that can be performed prior to initiating the ITT. The critical steps include handling and injecting mice without the use of a restraining device. Special attention should be given to the ambient noise, lighting, temperature and any movement in the room in which the ITT will be performed. It is advised that mice be transferred to this room at the start of the fasting period, so as to minimize exposure to changes in the environment immediately before the ITT begins. It is also important for the investigator to remain calm as this may impact anxiety-related behaviors in mice. In db/db mice, we found that the acclimation procedure abolished the rise in glucose levels between 0 and 15 min during the ITT. Further, in non-diabetic control mice, the acclimation procedure resulted in a steeper decline in glucose levels between 0 and 15 min during the ITT. Importantly, non-diabetic control mice that had not been acclimated did not exhibit a rise in glucose levels at the onset of the ITT, yet upon acclimation they still responded favorably by

displaying a greater rate of glucose disposal. The latter suggests that stress during an ITT may not always manifest itself as elevated glucose levels above baseline, however taking steps to mitigate stress may avoid misinterpretation of the data.

The data support the implementation of an acclimation period prior to performing an ITT. However, the method proposed has some limitations. Perhaps the most obvious of these is the need to handle the mice. Specifically, the protocol involves mice being picked up by the tail. A previous study compared glucose levels using this handling method with the "cup" method, which involves being scooped up and being free to roam in the handler's open gloved hands without direct physical restraint. It was found that the cup method resulted in lower fasting glucose levels and improved glucose tolerance, as well as lower plasma corticosterone concentrations and reduced anxiety-like behaviors[15,16]. Thus, the method of mice being picked up by the tail may still elicit a stress response, though it is important to recognize that in general, handling positively habituates animals to human contact. Another limitation is the need to sample blood from the tail, which can itself induce stress. In a study comparing catecholamine levels in samples obtained via tail cutting versus artery sampling [17], catecholamines were higher in the former indicating that cut tail sampling can be stressful. However, this is only the case when large blood volumes (~100 μL) are sampled and the tail is massaged or squeezed. Indeed, when minimal volumes of blood (~5 μL) were sampled from the tail, catecholamine levels were not elevated[17]. Thus, given that the acclimation (and ITT) procedure only requires a small blood volume for glucometer readings, the cut tail method is a useful technique that potentially avoids a stress response. A drawback of the study that limits a more definitive assertion in this regard is the fact that stress hormones were not measured (which would have required a large blood volume).

As evidenced by the data, the acclimation procedure is useful for obtaining more reliable ITT data from both diabetic and non-diabetic mice. It is possible that additional refinements to the procedure will further lessen the impact of stress during an ITT. For example, during the acclimation period, we propose "mock" injections in which mice receive i.p. saline. It would be useful to know whether delivery of the saline is actually necessary, or whether mice can simply be handled and an empty syringe inserted intraperitoneally. Also, the number of times mice are handled, as well as the period between each handling, could be tested. Currently, the protocol indicates that they be handled twice during the acclimation period, with a 60 min interval between the start of each handling period. Additionally, we incorporate a 60 min recovery period from the last handling/injection to the start of the ITT. It remains to be determined whether a shorter or longer recovery period would further mitigate the stress-induced hyperglycemia we observe in db/db mice. Previously, it was reported that in response to stressful stimuli such as handling, noise and i.p. injections, rodents exhibit elevated plasma corticosterone levels for at least 2 hours[18], suggesting that perhaps a longer recovery period would be useful. Further, stress responses can differ amongst different strains of mice. For instance, tail handling can trigger seizures in susceptible strains[19]. In considering all of these variables, we propose that the precise conditions for acclimation prior to an ITT may need to be determined empirically by each investigator, using our protocol as a starting point.

In summary, we have described a method of acclimating mice to handling, injections and blood sampling prior to performing ITTs. We find that this significantly lowers stress-induced hyperglycemia and results in data that more accurately reflects insulin action.

ACKNOWLEDGMENTS:

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DISCLOSURES:

The authors have nothing to disclose.

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Figure 1

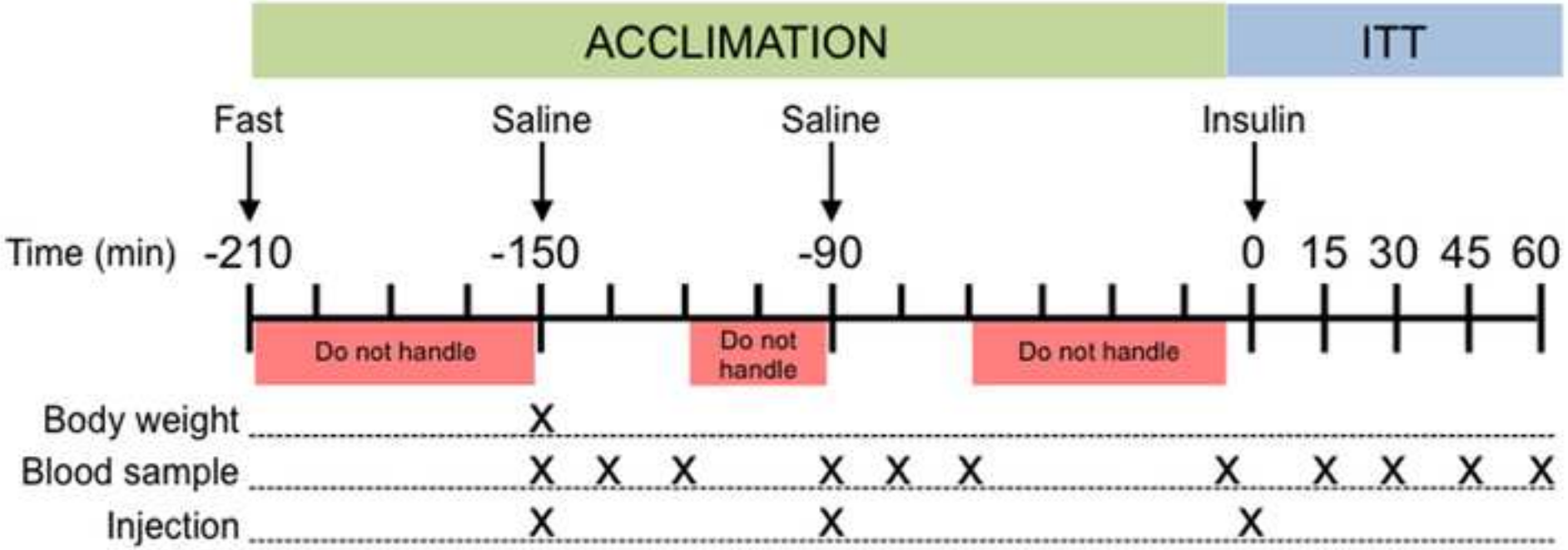

Figure 2

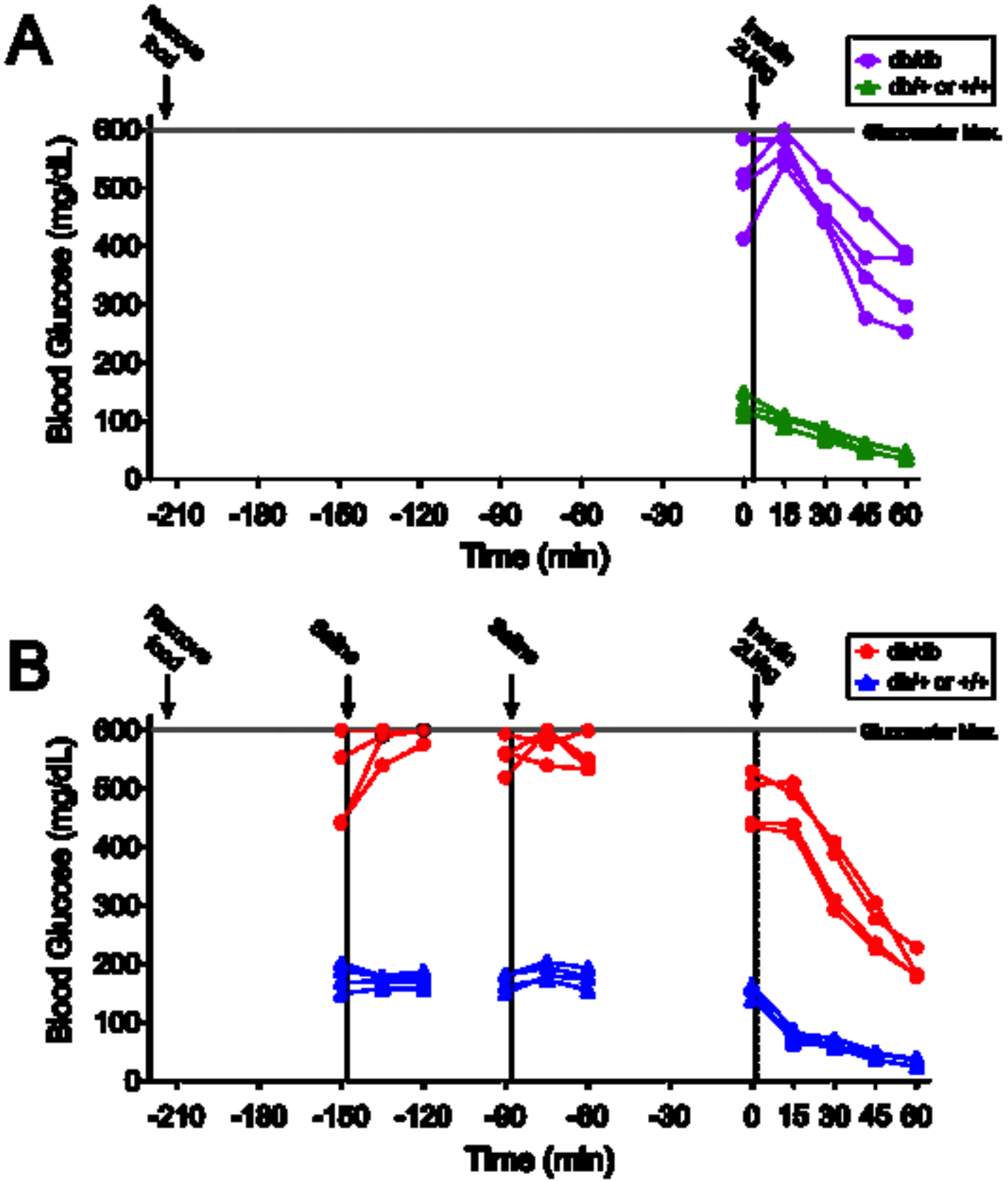

Figure 3

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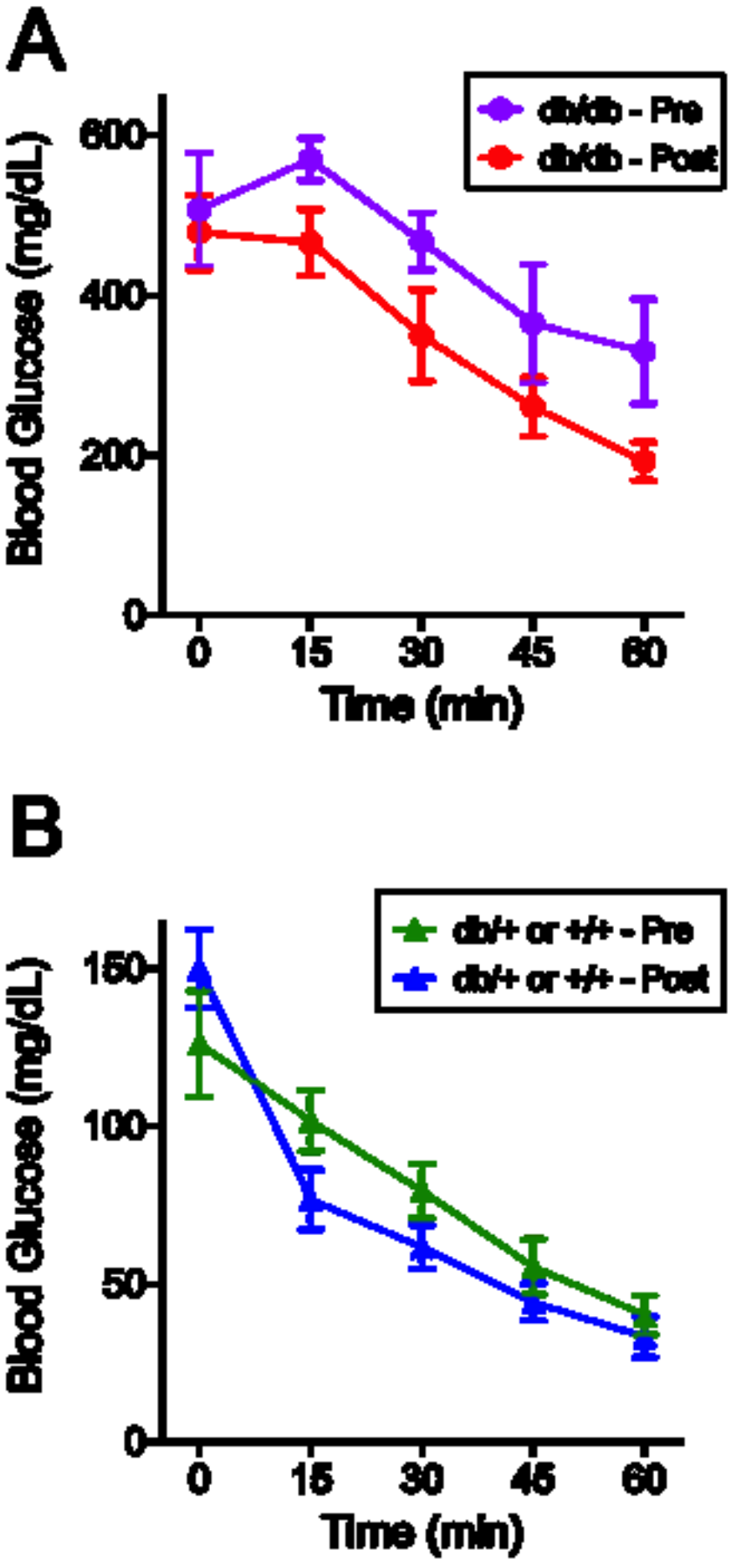

Name of Material/Equipment	Company	Catalog Number	Comments/Description
Dextrose-50	Pfizer Injectables	00409-6648-16	For use if mouse experiences hypoglycemia.
Gauze pads	Fisher Scientific	22037907	To dislodge blood clot on the tail tip.
Glucometer	Accu-Chek	M001_us	To measure blood glucose.
Gram scale			To measure body weight.
Insulin (Novolin R)	Novo Nordisk	0169-1833-11	For injection.
Insulin syringes	VWR	BD-329461	For injections.
Minute timer			
Sterile 20 G needle	VWR	BD-305175	For tail snip.
Sterile saline	Lifeshield	1261699	For injections.
Surgical scissors	Fine Science Tools	14088-10	For tail snip.
Test strips	Accu-Chek	06908217001_us	To measure blood glucose.

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Response to Reviewers

Please note that changes within the manuscript are shown in red font.

Reviewer #1:

1. The blood sugar in control mice during fasting seems to be a bit high? Due to stress?

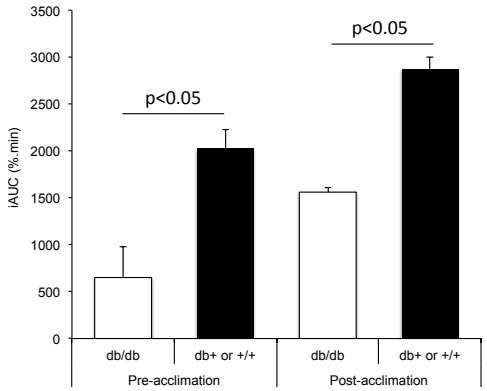
Our glucose data suggest fasted control mice experience some stress during handling prior to the insulin tolerance test (ITT). However, at the t=0 timepoint of the ITT, the blood glucose levels are lower than pre-ITT levels at -60 min (150±6 vs. 175±8 mg/dL; n=4, p=0.02), suggesting a reduced stress response following acclimation. These data are now highlighted in the results section of the manuscript. Also, in other studies from our lab where control mice of the same genotype are chronically catheterized and blood is sampled without handling (i.e. via the arterial catheter) in the conscious, unstressed state, we have found that blood glucose after a 6-hour fast is comparable to what is seen at t=0 in the present study (156±8 mg/dL, n=6). This suggests that if fasted control mice experience some stress, it is likely minimal after they have been acclimated to handling. While it is possible stress is not completely eliminated following acclimation, the relatively short duration of fasting (i.e. 3.5 hours) may be another reason fasting glucose levels are not lower than observed.

2. Statistics analysis results should indicate in the figure 3.

We have now performed statistics to determine whether the fall in glucose levels following insulin administration is significantly different between acclimated and non-acclimated mice. To do this, we compared the slopes from 0 to 15 minutes, since this 15-minute period most closely estimates insulin sensitivity, whereas counter-regulatory responses to hypoglycemia can occur beyond this timepoint. We found that the rate of glucose disappearance in the first 15 minutes is significantly different (p=0.002) between control mice that are acclimated and those that are not. In db/db mice, this is not the case, likely because (i) baseline glucose levels (at t=0) were highly variable amongst the four non-acclimated mice, and (ii) glucose levels had already decreased between t=-150 and -60 minutes in mice that underwent acclimation. The latter resulted in overall lower glucose levels during the ITT in acclimated versus non-acclimated db/db mice (p<0.05, 2-way ANOVA). Statistics have been described in the results section and added to the figure legends.

3. Insulin 2U/kg seems to be too strong for db/db because a possible "insulin resistance" only appeared within the first 15 min. If authors can demonstrate that this protocol provide a clear difference of insulin sensitivity between control and db/db mice, it will be very attractive for readers in the field of diabetes research.

Our study was not intended to compare glucose responses between control and db/db mice, but rather to demonstrate that acclimation of mice to handling can mitigate stress-induced hyperglycemia within a group of mice. That said, we have now performed statistical analysis to show that the glucose response to insulin (represented as inverse area under the curve, iAUC) is significantly different between control and db/db mice, regardless of whether they have undergone acclimation. We feel this is not surprising given that there is a large difference in insulin sensitivity between control and db/db mice. These data are shown below. Note that raw glucose data were first expressed as % of baseline, in order to adjust for the differing baseline glucose levels between control and db/db mice.



4. What time during a day is recommended for the protocol?
We recommend food withdrawal in the morning after the dark cycle has ended. Fasting time of day and duration should be consistent across groups of mice. For this protocol, food was removed between 0700 and 0800 hours, which is 1-2 hours after the light cycle begins. This information has been added to the protocol in the manuscript.

5. Can legends of each colored lines used in each Figures be added?
We have now added a legend to each figure to denote the different groups.

6. Strain of control mice in the demonstration?
The control mice were db/+ or +/+ littermates from Jackson Labs, where they are not genotyped to differentiate the two. We have now added this information to the Results section and figures.

Reviewer #2:
1. Interesting thought that corn cob bedding may be a carb source in mice. Is there a reference or any data to support this? If no, please soften the statement to conjecture.
The following reference has been added to support the statement regarding use of corncob bedding: Zahorsky-Reeves J, Castellani LW. Housing Mice on Corncob Bedding versus Hardwood Chip May Confound Research Models. Am Assoc Lab Anim Sci (10 October 2010). AALAS Scientific Session.
Also, data from our lab show that when compared to mice fasted overnight with CareFresh bedding, mice fasted overnight with corncob bedding have elevated glucose levels (CareFresh 163±12 vs. corncob 228±14 mg/dL; n=3, p=0.004). We have not added these data to the manuscript as they were derived from another cohort of C57BL/6 mice.

2. Section 2.2 it sounds like the tail is snipped while the mouse is suspended in the air. Is the mouse placed on a wire rack or other grippy surface? Please describe.
Mice are not suspended when the tail is snipped. They are placed on a flat tabletop surface. This information has been added to the protocol in the manuscript.

3. Line 234 in the discussion hyperglycemia should not be in quotes, it is real, just not due to the injected substance.
We agree and have now removed the quotation marks.

4. Line 241 in the discussion a failure of glucose levels to fall over the entire period may be more related to insulin dose or insulin resistance than stress, recommend soften that statement.
We have now edited the sentence to say: “Finally, an additional 10 studies failed to observe a decrease in glucose levels following insulin administration in db/db mice over the course of the ITT, though this may have been related to the dose of insulin used or a state of marked insulin resistance in the mice”.

5. Line 258 do you mean elevated above baseline?
Yes, we have now modified this statement for clarity.

6. Line 304 it's not really valid to say that this method more accurately reflects insulin sensitivity since gold standard wasn't measured. Perhaps change to more accurately reflects insulin action?
We agree and have now made the suggested change to the text.

Reviewer #3:
Figure 3 - Can the authors provide statistics to support the conclusion that the fall in glucose level between procedures are significant?
We have now performed statistics to determine whether the fall in glucose levels following insulin administration is significantly different between acclimated and non-acclimated mice. To do this, we compared the slopes from 0 to 15 minutes, since this 15-minute period most closely estimates insulin sensitivity, whereas counter-regulatory responses to hypoglycemia can occur beyond this timepoint. We found that the rate of

glucose disappearance in the first 15 minutes is significantly different ($p=0.002$) between control mice that are acclimated and those that are not. In db/db mice, this is not the case, likely because (i) baseline glucose levels (at t=0) were highly variable amongst the four non-acclimated mice, and (ii) glucose levels had already decreased between t=-150 and -60 minutes in mice that underwent acclimation. The latter resulted in overall lower glucose levels during the ITT in acclimated versus non-acclimated db/db mice ($p<0.05$, 2-way ANOVA). Statistics have been described in the results section and added to the figure legends.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have now proofread our manuscript to ensure there are no spelling or grammar issues.