LAB_057 Insulin Tolerance Test in Mice

I. OBJECTIVE

To describe the general procedural expectation for performing safe, effective, and humane insulin tolerance tests (ITT) in mice.

II. DEFINITIONS

Competent - "the consistent application of knowledge and skill to the standard of performance required regarding the care and use of animals. It embodies the ability to transfer and apply knowledge and skill to new situations and environments." (NHMRC, 2013).

Insulin Tolerance Test (ITT) – A test performed to evaluate insulin sensitivity by monitoring glucose clearance from the blood over time following administration of an injection of insulin. Poor sensitivity to insulin results in a reduced rate of glucose clearance. As a research tool, this test is useful in studying diseases such as metabolic syndrome and diabetes mellitus.

International Unit (IU) – in pharmacology, this is a standardised unit of measurement relative to the expected effect, as compared to the mass of a substance. For insulin, IU is the most commonly used unit of measurement.

High Fat Diet (HFD) - A diet which contains 45-60% fat, as compared to standard rodent chow diet, which contains ~10% fat.

III. COMMENTS / RECOMMENDATIONS

- Relative to animal ethics applications, when using this SOP, the following must be described in the individual ethics application: insulin type and dose, method of blood collection, and any intended variation to this SOP.
- ITT data outcomes, and reproducibility of these results, are significantly influenced by suboptimal and inconsistent procedures. It is critically important for the scientific validity of data obtained that ITT is well planned, and performed in a precise and controlled manner, as outlined in this SOP, and summarised within textbox 1, within the reference information section.
- Matching of animal-groups is an important consideration as age, sex, strain, body weight, reproductive status, and health status, among others animal characteristics, are all known to influence insulin sensitivity and glucose tolerance.
- Stress significantly impacts glucose metabolism. The test environment should be managed as a "low stress" environment, free from uncontrolled external stimuli such as human traffic, unnecessary noise, and intense lighting. It is recommended in the days to weeks prior to commencing experimentation that the mice are trained to tunnel handling or other low-stress handling techniques by the person/s that will be completing the ITT. This period of acclimatisation will enable more efficient handling, and reduced stress for the mice throughout the procedure (Gouveia *et al.*, 2017; Henderson *et al.*, 2020).
- All other stimuli must be controlled as much as possible. For example, light/dark cycles, animal feed, ventilation, caging systems, and environmental enrichment should all be standardised. Note: glucose clearance is variable dependent on by time of day, and light/dark phase (Carroll *et al.*, 1973; la Fleur *et al.*, 2001); study design should account for, and aim to control, this potential variability.
- "Diseased" or "sick" mice (i.e. animals with any symptoms, exposure history, or diagnostic results which indicate sub-optimal health) should not be used within ITT procedures, unless that disease state is considered part of the study design (e.g. pharmaceutically induced diabetes mellitus, phenotypic SOD1 mice).

Conditions:

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- The type and dose rate of insulin administered must be established relative to the experimental design. Note, the different types of insulin are not interchangeable, they often require different doses and will cause different rates and duration of glucose clearance. In laboratory animal research, literature commonly cites the use of short-acting insulin (e.g., Actrapid®, Humulin®). In this setting short-acting insulin is often dosed at 0.5 – 0.75IU/kg. This dose rate should be calibrated relative to the individual mouse's characteristics, including gross body mass, or lean body mass (for obese animals), any expected insulin resistant (e.g., from type II diabetes mellitus) or strain differences.
- Fasting is a standard component of ITT. Prior to insulin administration mice are usually fasted for 2-6 hours (NB: fasting from food only, mice are still permitted free access to water). Fasting for >6 hours increases the risk of critical hypoglycaemia and should be avoided in this procedure. Where fasting >6 hrs is intended to occur for the purpose of performing ITT, the reviewing AEC will expect specific justification as to why this variation is required and why it should be considered ethically acceptable.
- Generally, anaesthetic agents should not be used to facilitate ITT as their use can potentially lead to confounding of experimental data. This is particularly relevant when using *LAB_013 Blood Collection – Tail Tip (Amputation) Bleed in Rats and Mice* to collect blood for ITT (i.e. anaesthesia should not be used to perform tail tip amputation when conducting ITT)

IV. EQUIPMENT

- Personal Protective Equipment (PPE)
  Requirements vary dependent on facility and the type of work being conducted.
- Permanent Marker
- Disinfectant (e.g. 70% ethanol)
- Electronic weigh scales
- Local anaesthetic ointment (e.g. Emla® cream)
- Heat source, e.g. heat mat, regulated warming box, or warm water bath,
  As per the relevant blood collection SOP, listed under step 5
- Clean restraint tubes (appropriate size and type)
  As per LAB_006 Handling and Restraint in Mice and Neonates
- Venepuncture instrument/tool, e.g. sterile lancet, number 11 scalpel blade, 25 - 30G needle
- Blood collection tubes (if not collecting blood directly onto the glucose test strip)
- Timer / stopwatch or clock
- Insulin stock solution (e.g. Actrapid 100 IU/mL, diluted in sterile saline to 1IU/mL)
- Insulin syringes (with 25-30G needle swaged)
- Gauze swabs
- Glucometer and compatible glucose test strips
- Sharp's container and clinical waste bin
- 10-25% Glucose solution (sterile, innocuous vehicle e.g. saline or PBS) <on hand for use in the event of critical hypoglycaemia>

V. PREPARATION

- Check AEC approvals, procedural documents, and animal identification, to ensure that the correct animals and procedures (including personnel and location details) have been selected for the scheduled work.
- Ensure appropriate experimental record sheets are organised (see table 1 for an example template, within the reference information section).

- Ensure the glucometer is functioning appropriately and 'ready to go' (e.g. there are sufficient numbers of compatible test strips available, and the device has sufficient battery charge).
- Establish what should occur in the event of critical hypoglycaemia, e.g., immediate euthanasia vs. therapeutic treatment with glucose.

VI. PROCEDURE

1. Measure and record the gross body weight of each mouse. Animals may be marked at the base of the tail using the permanent marker to assist with identification.

   Animal handling is performed in accordance with LAB_006 Handling and Restraint in Mice and Neonates.

2. Place the mice in a clean cage with routine access to water, but no access to food. Commence fasting of the mice for 2 to 6 hrs (see comments/ recommendations for guidance on fasting duration).

3. Measure and record gross body weight of each mouse, post fasting.

   At this time topical local anaesthetic ointment (e.g. Emla® cream) may be applied to the venepuncture site and the mice may be warmed, as per the relevant blood collection SOP, listed under step 5

4. Calculate and record the amount of insulin to be administered to each animal, relative to body weight.
   - ITT, insulin (Actrapid) dose = 0.5 – 0.75 IU/kg
     To achieve 0.5IU/kg body mass, using 1IU/mL insulin stock solution

     Volume of insulin stock solution for injection (µL) = 0.5(IU) x body weight(kg) / 1(IU/mL)

     For example, a 25g mouse will require an injection volume of 12.5µL

   Ensure injection volumes calculated do not exceed the maximum limits as prescribed within the relevant SOPs, listed under step 6.

5. Restrain the first mouse as required and collect a blood sample. This initial blood sample is used to measure and record the fasting basal glucose level. Blood samples are collected directly onto a glucose test strip for immediate assessment. Alternatively, a capillary tube or other blood collection tube may be used.

   Blood collection may be performed via any of the methods listed below:

   - *LAB_013 Blood Collection – Tail Tip (Amputation) Bleed in Rats and Mice (without anaesthesia)*
   - *LAB_019 Blood Collection - Tail Bleed in Rats and Mice*
   - *LAB_020 Blood Collection – Facial Bleed (Sub-Mandibular) in Mice*
   - *LAB_036 Blood Collection – Saphenous Vein in Mice*
   - *LAB_037 Blood Collection – Pedal Vein in Bleed Mice*
   - *Blood collection via an indwelling cannula*

   Following any blood collection method, ensure the collection site has stopped bleeding. If ongoing bleeding is observed gentle pressure using a gauze swab may be applied. In the case of tail tip amputation, Emla® cream applied directly to the amputated tip can act as a useful occlusive layer/bandage.

6. Immediately following step 5, administer the predetermined insulin dose (identified in step 4), via LAB_028 Injections - Intra-peritoneal (IP) in Mice, Rats and Neonates.

7. Record the time of blood collection and insulin administration (from steps 5 and 6, respectively) as the "start time" on the experimental record sheet (and start a timer accordingly).

   Often multiple mice will be undergoing ITT on the one day, at the same time. Careful time management is required to ensure the blood collection points are accurately reflective of the scheduled time points for each mouse. As such, individual animals should be staggered (e.g., using 1–5-minute intervals).

8. Monitor animals for hypoglycaemia (through to step 12) and if observed take immediate action to either a) immediately euthanise the animal/s, or b) administer therapeutic treatment using glucose.

   Critical hypoglycaemia is recognisable by any of the following symptoms: weakness, lethargy, apathy, tremors, heightened anxiety, loss of consciousness, seizure/convulsions; or a blood glucose level <2mmol/L (<36mg/dL).

   a) immediately euthanasia – as per any of the approved methods for mice, see SOPs

   b) therapeutic treatment with glucose, via LAB_028 Injections - Intra-peritoneal (IP) in Mice, Rats and Neonates.

      This 'rescue' intervention is only appropriate if the animal's wellbeing is not compromised and if the animal has significant value for use in subsequent testing (e.g. subsequent behavioural testing).

      Glucose dose = 0.5 – 1 mg/g

      To achieve 1mg/g body mass, using 20% glucose solution:

      Volume of glucose solution for injection (mL) = 1(mg) x body weight(g) / 200(mg/mL)

      For example, a 25g mouse will require an injection volume of 125µL

      Ensure injection volumes calculated do not exceed the maximum limits as prescribed within the relevant SOPs, listed under step 6.

   If critical hypoglycaemia occurs within the model refer to guidance, relative to unexpected adverse events, provided on the animal ethics webpage.

9. Collect serial blood samples at predetermined timepoints post insulin administration. Ensure each timepoint and sample result is recorded on the experimental record sheet.

   a) These timepoints for blood collection generally include: 15, 30, 45, 60, 90, 120 minutes post insulin administration.

   b) The predetermined timepoints may vary between individual models, however, the number of blood collection points should not exceed 10 in total (inclusive of the initial blood collection for establishing the fasting basal glucose level);

   c) Collection volumes at each timepoint should remain <10uL;

      Larger blood volumes may be collected (e.g. 20uL) so long as the following point is observed (relative to total blood volume collected);

   d) The total volume of blood collected must be the minimum amount required for the project, should not exceed a "moderate bleed", and must not exceed a "major bleed" (see table 2, within the reference information section).

   Blood collection in rodents almost always causes some "wastage" (e.g. oozing or dripping of blood post venepuncture, extravasation of blood into the subcutaneous space). It is critical that this volume of wastage is estimated and accounted for when estimating the total blood loss to the animal.

10. At the end of the experiment return mice to their home cages with food and water and monitor their general behaviour and physical condition. If any signs of pain and distress are observed take immediate action as appropriate – for guidance refer to the animal ethics webpage.

11. Reassess the mice after at least 1 hour, before returning to routine monitoring protocols.

    It is recommended that these animals are then permitted at least a 24hr "respite" period, where they are not used for other procedures.

12. After the ITT procedure, stored blood samples may be assessed further using various assays (*ex vivo*). This testing may require specific blood handling and storage (e.g. collection of blood plasma and storage at ≤ (-)20°C, until analysis).

VII. REFERENCE INFORMATION

TEXTBOX 1 | Summary of General Experimental Considerations, relative to ITT and Glucose Tolerance Test (GTT) (image source: Benedé-Ubieto *et al.*, 2020). *In the context of this SOP, please consider the following comments:*

- "same gender", should be considered to read as "sex matched"
- minimum animal numbers per group, should be established based on power analysis
- *GTT is not considered part of this SOP. For information relative to this procedure please refer to LAB_05f Glucose Tolerance Test (GTT) in Mice.*

Experimental Pre-Settings

- Quiet and stress-free environment
- Standardized and persistent conditions through the whole experimental period (i.e. time of fasting, route of administration, dosage of glucose/Insulin, brand of glucometer)

Quick Assay Procedure

- Pre – fasting for 6 or 12 hrs
- Measure mice body weight after fasting
- Calculate the amount of glucose and insulin needed
  - GTT. Volume for injection = 7.5 x Body weight. From 20% Glucose stock solution.
  - ITT. Volume for injection = 3 x Body weight. From 0.25 UI/mL Insulin in saline solution (Ex. 9.975 mL saline solution + 25 µL Insulin 100 UI/mL).
- Cut tail and measure basal glucose in the blood
- i.p. with a time-lapse between mice of 3 min.
- Repeat determination of blood glucose concentration after 15, 30, 60, 90 and 120 min.

Considerations for Minimize Intra-Group Differences

- Genetically identically inbred
- Similar age
- Same gender
- Minimum 5 animals per group
- Perfectly matched control group, preferentially littermates

TABLE 1 | Example experimental record sheet for conducting ITT.

Mouse ID#	BW (g)	Insulin (µL)*	Time of glucose injection	Glucose levels (mg/dL)						
				0min	15min	30min	45min	60min	90min	120min
*Insulin type: _____, and concentration _____ (IU/mL)										

TABLE 2| Recommended blood collection volumes based on a mouse's live body weight (NHMRC 2008).
The total amount of blood loss from any blood collection procedure must take into account the sample volume collected as well as any circumstantial bleeding (e.g. prolonged bleeding post venepuncture).

Mouse Weight	TOTAL BLOOD VOLUME (TBV) *[equates to 5-7% of body weight]*	Minor bleed *(<7.5% of TBV)*	Moderate bleed *(7.5-10% of TBV)*	Major Bleed *(10-15% of TBV)*
Recovery period required between bleeds, relative to volume collected:		1 week recovery	2 weeks recovery	3 weeks recovery
18g	1.2mL	<90uL	90-120uL	120-180uL
22g	1.5mL	<115uL	115-150uL	150-225uL
26g	1.8mL	<140uL	140-180uL	180-270uL

VIII. BIBLIOGRAPHY

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2	LBM	01/12/2022	01/12/2025

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