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Comparison of blood sampling methods for plasma corticosterone measurements in mice associated with minimal stress-related artefacts

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ABSTRACT

Accurate measurement of circulating glucocorticoid concentrations in rodents is often hampered by the stress-related activation of the hypothalamic-pituitaryadrenal axis during animal handling. The present study aims to identify methods of blood collection associated with minimal stress and thus artificial increases in plasma glucocorticoid levels. Using two strains of mice, we evaluated common laboratory methods of non-terminal (tail blood sampling with or without restraint; retro-orbital puncture) and terminal blood collection (cardiac puncture) and their immediate and prolonged effect on plasma corticosterone levels.

Compared to retro-orbital and cardiac puncture, mice from both the unrestrained and restrained tail snip collection groups displayed the lowest plasma corticosterone levels in both mouse strains. Plasma corticosterone levels in samples obtained from retro-orbital and cardiac puncture collection were up to twenty times higher than those measured in mice undergoing blood collection via tail snip. Repeat tail snip collections (every 30 min for 120 min, or once after 120 min) revealed sustained hypercortisolaemia, compared to the initial collection.

We conclude that blood sampling via tail snip without restraint remains the gold-standard method of collection that is associated with minimal stress-related artefacts and hence feasible for single time point corticosterone analyses.

1. Introduction

When investigating the hypothalamic-pituitaryadrenal (HPA) axis in humans and animals, it is important to minimise variables that may affect experimental results, in particular circulating glucocorticoid levels. Rodents are exceptionally sensitive to changes in the environment, pain, manual handling and restraint. All of these are part of routine blood collection procedures and cause stress, therefore affecting HPA axis activity [1,2]. As a result, plasma levels of corticosterone, the predominant glucocorticoid in rodents, are often artificially exaggerated and results are unreliable.

Although there are methods to measure corticosterone levels that are non-invasive and require minimal direct interaction/disturbance for collection, several limitations hinder their routine use with laboratory animals as an alternative to blood sampling. For example, measures of cortisol in hair samples have been developed [3–5], however this sampling method is only suitable for assessment of chronic conditions, as well as animals that have sufficient hair length for analysis. Corticosterone concentrations can also be determined in faecal samples, however faecal measures are currently only used to complement blood corticosterone measures as the delay between corticosterone changes in the blood and the manifestation of these changes in faeces is as yet unknown but likely to be significant [6]. Moreover, both these methods are not sufficiently sensitive for assessing minor or acute systemic corticosterone level changes (e.g. across minutes or hours).

As blood sampling remains the most commonly used method for the assessment of HPA axis activity, and as there is currently no appropriate alternative method other than blood sampling for assessing acute changes in corticosterone levels in mice, it is pivotal to select a method of blood collection that is associated with minimal stress-related artefacts. Thus, the aim of the present study was to assess and compare plasma corticosterone concentrations in samples obtained from common laboratory methods of non-terminal (tail blood snipping with or without restraint; retro-orbital puncture) and terminal blood collection (cardiac puncture). Since there may be strain differences in corticosterone responses in mice [7], we compared blood sampling techniques in two commonly used strains of mice.

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2. Experimental

2.1. Animals

Outbred CD-1 Swiss White mice (CD-1) and inbred C57BL/6 male mice were used for all experiments. Prior to experimental procedures, mice were housed under standard animal laboratory conditions (3–5/cage) on a 12:12 h light–dark cycle, with *ad libitum* access to standard rodent chow and water at the animal facility of the ANZAC Research Institute. All experiments were conducted with protocols approved by the institutional Animal Ethics Committee.

Seven-week-old male mice were acclimatised overnight to the room where the blood collection would take place. All blood collections were performed between 10:00 and 12:00 h, when corticosterone levels are expected to be close to the circadian nadir in rodents. In order to minimise differences and potential stress on animals between samplings, the same person handled the same mouse for the course of the experiment.

2.2. Blood collection methods

We tested four different blood collection methods (4–6 mice/group): Unrestrained tail snip, restrained tail snip, retro-orbital puncture (all non-terminal) and cardiac puncture (terminal). All procedures were performed by staff with more than 5 years of animal handling experience and no complications arose from any of the interventions.

2.2.1. Unrestrained tail snip

Mice were free to move around while the distal tip (1–1.5 mm) of the tail was removed using a sterile scalpel blade. Blood (15–20 μL) was then collected by gently massaging the tail from the base to the tip into heparinised tubes (Microvette® CB 300, Sarstedt, Nümbrecht, Germany). The volume of blood collected was limited to the volume required for corticosterone analysis and pressure was gently applied to stop any bleeding before returning to cage. This procedure took less than 1 min and the initial sample was taken as baseline. Serial samples were collected 30, 60, 90 and 120 min after the initial collection by massaging the base to the tip of the tail. Any scab formed on the tip of the tail (usually at the last time point) was removed gently using sterile gauze.

2.2.2. Restrained tail snip

At the time of blood sampling, mice were placed in a restraining tube and blood was drawn as described above. Mice were restrained for less than 2 min. Pressure was gently applied to stop any bleeding before returning to cage. A second blood collection was performed 120 min after the initial collection using the restraining tube.

2.2.3. Retro-orbital puncture

On the day of blood collection, mice were put under general anaesthesia by intraperitoneal injection of a 0.075% ketamine and 0.01% xylazine cocktail. Blood (< 100 μL) was collected by retro-orbital puncture, using micro-haematocrit capillary tubes (Livingstone, Rosebery, NSW, Australia). Once the tube was withdrawn, slight pressure was applied on the eyelid with gauze to prevent further bleeding.

2.2.4. Cardiac puncture (terminal)

On the day of blood collection, mice were put under general anaesthesia by intraperitoneal injection of a 0.075% ketamine and 0.01% xylazine cocktail. Blood was collected by standard cardiac puncture method, followed by cervical dislocation.

Tail snip blood was also collected from anaesthetised mice (15–20 μL) as an anaesthetic experimental control group.

2.3. Plasma corticosterone measurements

All blood samples were centrifuged at 2000*g* for 5 min at room temperature and stored at −80 °C until further analysis. Plasma corticosterone concentrations were measured using an enzyme immunoassay kit (Arbor Assays, Ann Arbor, USA), following the manufacturer's instructions. This kit has intra- and inter-assay coefficients of variation of 6.3 and 7.5% respectively, at a concentration of 7.2 nmol/L and a limit of detection of 0.05 nmol/L.

2.4. Statistical analysis

Results are expressed as mean ± standard error of mean (SEM). For time response measurements, a one-way analysis of variance (ANOVA), repeated measures was performed, followed by Dunnett's multiple comparisons test (compared to 0 min). When comparing different methods of blood collection, one-way ANOVA, followed by Tukey's multiple comparisons post-hoc test was performed. All calculations were performed using GraphPad Prism 7 software (GraphPad Software Inc., La Jolla, USA).

3. Results

3.1. Corticosterone levels using different collection methods

Compared to anaesthetised tail snip, retro-orbital and cardiac puncture, both unrestrained and restrained tail snip were associated with the lowest plasma corticosterone levels (Fig. 1A & B). Anaesthetised tail snip, retro-orbital and cardiac puncture resulted plasma corticosterone levels up to twenty times higher than those measured in mice undergoing blood collection via tail snip in conscious mice.

3.2. Unrestrained tail snip – time course

As unrestrained, freely moving mice appeared undisturbed by the collection process, we asked the question whether corticosterone levels would remain low with repeated collections, such as those required for insulin tolerance and similar time-dependent tests that only require a small volume of blood to be collected (< 20 μL). Following tail snip at time 0, further blood samples were therefore obtained at 30-minute intervals for 120 min. In CD-1 mice, plasma corticosterone concentrations at the 30-minute time point had increased to 10-fold the levels seen after the initial blood collection. Concentrations had fallen slightly at 90 and 120 min, but remained significantly higher than the levels measured at initial blood collection (Fig. 2A). C57BL/6 mice also showed a significant increase in corticosterone after 30 min, which remained at similar levels for the duration of the study (Fig. 2B).

3.3. Restrained tail snip – repeat collection

To assess whether mice become acclimatized to restraining, a second blood collection was taken 120 min later, again using the restraining tube. As seen in Fig. 3A, compared to the initial corticosterone level, CD-1 mice showed a ten-fold increase in their plasma corticosterone levels. C57BL/6 mice displayed a similar pattern, albeit to a lesser degree than CD-1 mice (Fig. 3B).

4. Discussion

This study compared different methods of blood collection to determine the optimal method that would cause least stress-related changes in plasma corticosterone levels due to handling. We found that plasma corticosterone levels in unrestrained, freely moving mice undergoing tail blood sampling were significantly lower than in mice subjected to tail snip (anaesthetised), retro-orbital or cardiac puncture.

The sampling methods resulting in the highest plasma