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Comparison of corticosterone responses to acute stress in mice following different serial blood collection methods

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Abstract

Background. Accurate evaluation of glucocorticoid concentrations during serial blood collection in rodents is often hampered by the stress response elicited by the procedure itself. The optimal method to minimize stress and impact on animal welfare remains debated.

Methods. Hence, we compared corticosterone concentrations in adult mice serially bled by using the retro-orbital sinus puncture or the tail vein incision methods, either with or without exposure to an acute restraint stress.

Results. Corticosterone concentrations were significantly affected by the sampling method, with higher peaks and sustained hypercortisolemia in mice bled with the retro-orbital sinus puncture, pointing to the tail vein incision method as preferable for serial blood collections. Mice bled using the tail vein incision reached similar corticosterone peaks regardless of exposure to acute stress.

Conclusions. Our findings suggest that tail vein incision can be used to evaluate neuroendocrine reactivity without exposing mice to restraint procedures. This would improve animal welfare practices in experimental protocols.

Key words

- restraint stress
- glucocorticoid
- retro-orbital sinus
- tail vein
- animal welfare

INTRODUCTION

When studying the hypothalamic-pituitary-adrenal (HPA) axis, it is crucial to minimize variables that could influence experimental outcomes, in particular glucocorticoid levels. Rodents are highly sensitive to environmental changes, physical handling, restraint and pain [1, 2]. These factors, which are inherent to blood collection procedures, may impact HPA axis activity, affecting results of blood assays and compromising animal welfare. Consequently, plasma concentrations of corticosterone, the main glucocorticoid in rodents, are frequently artificially elevated, creating inaccurate experimental outcomes [3].

Methods for measuring corticosterone concentrations that cause minimal disturbance to the animals during collection pose limitations that prevent their use as an alternative to blood sampling. For instance, there is a significant, not easily quantifiable, delay between the appearance of corticosterone in feces and changes in blood corticosterone. Similarly, corticosterone levels in hair samples can only inform about chronic conditions. Furthermore, neither of these two non-invasive methods is sensitive enough to detect acute (e.g., over minutes/hours) or minor corticosterone fluctuations [3].

Since there is no suitable alternative to blood sampling for evaluating acute changes in corticosterone levels in rodents, it is crucial to choose a collection method that lessens stress-related artifacts and minimizes pain and distress, as ethically and legally required by the principle of refinement [4]. Multiple sites are routinely used for blood collection, including the retro-orbital (or retro-bulbar) sinus/plexus, lateral tail vein, jugular vein, saphenous vein, heart, sublingual vein and facial (or submandibular) vein. As for the lateral tail vein, blood droplets can be obtained through different methods, including tail vein puncture (i.e., inserting a needle into the vein), tail vein incision (i.e., using a blade, also known as tail nick) and tail snip (i.e., amputation of the tip of the tail, also known as tail clip). These tail sampling procedures involve different levels of handling/restraint and potentially different levels of pain, which can confound the results of corticosterone analyses and impact animal welfare [5, 6].

The retro-orbital sinus/plexus method is widely performed as it allows a skilled experimenter to rapidly obtain blood samples of good quality (e.g., not subjected to hemolysis); however, multiple side effects have also been reported following the application of this tech-

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nique. For example, Mahl and co-authors [7] reported, in rats, higher degree of tissue damage and stress associated with blood sampling from the retro-orbital plexus than from the sublingual vein (both performed after isoflurane anesthesia). However, Teilmann and co-authors [8], who compared, in mice, retro-orbital sinus and facial vein punctures (both without anesthesia), found that the impact of the latter was more severe, as indicated by increased plasma corticosterone levels, while the level of tissue trauma was comparable. Further, Tsai and co-authors [9] suggested that, in mice, retro-orbital bleeding (without anesthesia) causes the least stress (also compared to tail vein puncture), whereas jugular vein bleeding and facial vein bleeding cause the most stress and saphenous vein bleeding causes the most lasting damage. Hence, the advantages and disadvantages of this method remain controversial.

Although several studies have compared the different blood collection methods and the associated effects on corticosterone levels, most consider only one or two time points. Only few comparative studies on serial blood collection have been conducted. Frohlich and co-authors [10] compared serial blood collection (once weekly for 6 consecutive weeks) by retro-orbital (after isoflurane anesthesia) and facial vein (without anesthesia) methods in mice and found that the latter caused substantial morbidity and mortality compared with the former. Opposite results were found by Jo and co-authors [11] using the same collection methods, under the same anesthesia regimen, but with different timing (on alternate days for 2 weeks).

In the present study, we compared plasma corticosterone levels during serial blood collection by the retro-orbital sinus puncture and tail incision methods. The latter is a commonly used alternative, which poses different advantages (e.g., light handling) and drawbacks (e.g., poor-quality samples). It also allows the blood flow to be started and stopped easily, facilitating the collection of small volumes of blood (typically 30-40 µl), a crucial aspect when it is necessary to take repeated samples from the same subject at multiple time points over few hours [6]. Conversely, when applying the retro-orbital puncture technique, it is fundamental to carefully monitor the amount of blood drawn in order not to exceed the recommended 10% circulating blood volume (NC3Rs Guidelines 2021, https://nc3rs.org.uk; NIH Guidelines 2022, https://oacu.oir.nih.gov).

The tail incision method is applied in unrestrained, freely moving mice, which appear almost undisturbed by the collection process. Many investigators favor this technique in behavioral studies as it is often considered a stress-free procedure [12, 6]. Hence, the first objective of the present study was to assess whether corticosterone levels would remain low with repeated collections over 2 h.

To study the reactivity of the HPA axis, after baseline blood sampling, animals are generally exposed to an acute stress (usually restraint stress) [13] and then subjected to additional blood collections over few hours to assess corticosterone peak concentrations and time course. However, to our knowledge, there are no comparative studies that assess corticosterone response following acute restraint stress using different serial blood collection methods. Hence, the second objective of the present study was to ascertain whether stress hormone profiles (in terms of peak responses and/or return to baseline) following restraint stress were affected by the sampling procedure, by comparing the retro-orbital sinus puncture and the tail incision methods.

MATERIALS AND METHODS

Ethics statement

All experimental procedures were approved by Institutional Animal Survey Board on behalf of the Italian Ministry of Health (license n. 409/2018-PR) and performed in full accordance with the Directive 2010/63/EU on the protection of animals used for scientific purposes and Italian Law (D.lgs. 26/2014).

Animals and rearing conditions

Experimental subjects were 20 CD1 male mice of approximately twelve weeks of age provided by Charles River (Calco, Lecco, Italy). Mice were housed in pairs in 33×13×14 cm polycarbonate cages with metal tops and with sawdust bedding and left undisturbed for four weeks prior to the experiment.

All animals had *ad libitum* access to tap water and food (Mucedola, Settimo Milanese, Milan, Italy) and to environmental enrichment in the form of shelter material (Nestlets®, Ancare, Bellmore, New York, USA). Animals were housed in an air-conditioned room (temperature 22±1 °C, relative humidity 45±5%), on a 12-h reversed light-dark cycle (lights off at 7:00 am).

Experimental design

In order to investigate the impact of different blood collection methods on the physiological stress response (i.e., plasma corticosterone concentration), mice were subjected to the collection of small blood samples at different time points from either the tail (TAI group) or the retro-orbital sinus (ROS group). In addition, for each method, half of the animals, after baseline sampling, were exposed to an acute restraint stress procedure (ARS group) by placing them, for 25 min, in transparent conical polypropylene tubes (3.0 cm outer diameter, 11.5 cm length; 50 ml Falcon®, Corning, New York, USA), perforated in various points (including the cap) to allow breathing/transpiration and to position the tail; at the end of the restraint stress, mice were returned to their cages. The remaining half were not subjected to the stress procedure and were therefore returned to their home-cages immediately after baseline sampling (CTRL group).

Thus, mice were randomly divided into four groups: i) mice bled from the tail and returned to the home-cage after baseline sampling (TAI-CTRL group); ii) mice bled from the tail and exposed to the 25-min restraint stress (TAI-ARS group); iii) mice bled from the retro-orbital sinus and returned to the home-cage after baseline sampling (ROS-CTRL group); iv) mice bled from the retro-orbital sinus and exposed to the 25-min restraint stress (ROS-ARS group).

Blood collection

Two mice were simultaneously taken to an adjacent room by two skilled experimenters and bled (t0) by either method (see below for details). The time elapsed between the experimenter entering the facility room and the completion of baseline blood sampling was less than 3 min [5]. After 25 minutes mice were bled again (t25) and then relocated to their home-cage. Additional samples were taken 35 min (t60) and 95 min (t120) later. To prevent potential confounders (e.g., corticosterone diurnal fluctuations, variations in technical expertise), testing was performed within a 2-hour time window (between 10:00 am and 12:00 am), simultaneously in all mice and by experimenters with extensive experience, ensuring a high level of technical proficiency and reproducibility.

For each sampling, approximately 30-45 µl blood was collected; hence, the total volume collected over 2 h did not exceed the recommended 10% of the circulating blood volume that can be safely removed at one time (ranging from 110-140 µl for a 20 g mouse to 170-210 µl for a 30 g mouse; NC3Rs Guidelines 2021, https://nc3rs.org.uk; NIH Guidelines 2022, https://oacu.oir.nih.gov).

Blood sampling from the tail

During each sampling, the animal was placed on the standard metal cage's lid and let free to move [6, 14, 15]. For baseline sampling (t0), the experimenter made a small nick (approximately 2 mm wide×0.5 mm deep) in the tail with a callus cutter blade (Credo, Haan, Germany), perpendicular to the tail vein, approximately 2 cm from the tip of the tail. Blood droplets were directly collected into capillary tubes (Microvette® 100 EDTA K3E, 100 µl, ref. 20.1278, Sarstedt AG & Co. KG, Nümbrecht, Germany). The subsequent sample (t25) was usually collected from the same tail incision, after gently removing the clot. Additional samples (t60 and t120) were generally collected from incisions at different locations, working towards the base of the tail in 0.5 cm increments. The blood flow was encouraged by gently stroking the tail and, usually, blood flow stopped spontaneously when stroking was stopped.

Blood sampling from the retro-orbital sinus

During each sampling, following application of a drop of ophthalmic anesthetic, the animal was held with the nondominant hand using the thumb and forefinger, and the skin around the eye was pulled taut [16]. A capillary tube (disposable glass Pasteur pipettes, VOLAC®, ref. D810) was inserted into the medial canthus of the eye (30-degree angle to the nose) through a slight thumb pressure and twisting motion that was enough to puncture the tissue and enter the orbital sinus. Once the required volume of blood was collected, the capillary tube was gently removed. Bleeding was stopped by applying gentle finger pressure over the puncture site. This procedure was repeated for all four samples (t0, t25, t60, t120). From the pipette, blood was immediately transferred into EDTA-coated tubes (Microvette® 100 EDTA K3E, 100 µl, ref. 20.1278, Sarstedt AG & Co. KG, Nümbrecht, Germany).

Plasma corticosterone measurement

Samples were cool centrifuged (2,500 rpm, 20 min at +4 °C) and the plasma stored at –80 °C until assayed. Corticosterone concentration was measured using a commercially available Corticosterone Enzyme-Linked Immunosorbent Assay kit (ADI-900-097, Enzo Life Sciences, Farmingdale, New York, USA) as previously described [17].

Briefly, samples (6 µl) were analyzed in duplicate. Corticosterone concentrations were determined based on the corticosterone standard curve (range 32-20,000 pg/ml) incubated under similar conditions in each assay. Only data derived from duplicates with <20% coefficient of variation were included in the analysis. The sensitivity of the assay was 27.0 pg/ml. Light absorbance was read with a light absorption microplate reader (AMR-100, Hangzhou Allsheng Instruments, Hangzhou, China) at 405 nm. Data were elaborated by sigmoidal 4-parameter logistic curve fit using GraphPad Prism (GraphPad Software, San Diego, CA, USA).

Statistical analysis

Corticosterone concentrations at different time points were analyzed using repeated measures ANOVA, with "bleeding technique" (two levels: tail *vs* retro-orbital sinus) and "stress exposure" (two levels: control *vs* acute restraint stress) as between-subjects factors and repeated measures ("time", four levels time point: t0, t25, t60, t120) as within-subjects factor.

Total corticosterone secretion was calculated using the area under the curve with respect to ground (AUC_G) formula [18, 19] and was expressed in arbitrary units. The AUC_G was analyzed using ANOVA with "bleeding technique" (two levels: tail *vs* retro-orbital sinus) and "stress exposure" (two levels: control *vs* acute restraint stress) as between-subjects factors.

Multiple post hoc comparisons were performed using Tukey's post hoc test where appropriate [20]. All statistical analyses were conducted using the software StatView 5.0.1 (SAS Institute Inc., Cary, North Carolina, USA). Data are expressed as mean±standard error of the mean (SEM). Statistical significance threshold was set at $p \leq 0.05$.

RESULTS

As expected, all groups exhibited similar basal plasma corticosterone concentrations (t0). In the absence of a main effect of "stress exposure" ($F(1,16)=1.221$, $p=0.2856$), the repeated measures ANOVA yielded both a significant main effect of the "bleeding technique" ($F(1,16)=63.536$, $p<0.0001$) and a tendency towards a significant "stress exposure" by "bleeding technique" interaction ($F(1,16)=3.604$, $p=0.0758$).

Noticeably, the subsequent prompt physiological response ("time": $F(3,48)=79.747$, $p<0.0001$) was primarily induced by the stress associated with the bleeding procedure rather than by the exposure to the restraint stress ("bleeding technique"×"time": $F(3,48)=17.661$, $p<0.0001$; "stress exposure"×"time": $F(3,48)=1.179$, $p=0.3274$).

Multiple comparisons on the significant "stress exposure" by "bleeding technique" by "time" interaction

(F(3,48)=3.262, p=0.0293) evidenced that mice bled from the tail and mice bled from the retro-orbital sinus showed a different pattern of response over time, regardless of the stress exposure (*Figure 1*).

Specifically, TAI subjects showed an increase in corticosterone concentrations at either point t25 (CTRL) or t60 (ARS); such rise steadily declined at point t120 in both TAI groups. Moreover, visual data inspection suggested that while control subjects bled from the tail (TAI-CTRL) showed the highest corticosterone level at point t60, TAI-ARS mice appeared to show the corticosterone peak already at point t25, i.e., immediately after the end of the restraint procedure (*Figure 1*). By contrast, ROS subjects showed a significantly more pronounced corticosterone response compared to TAI mice at all time points (t25, t60, t120) in CTRL mice and at points t60 and t120 in mice subjected to the restraint procedure (ARS). No significant differences were found between ROS-CTRL and ROS-ARS groups. In addition, in both ROS groups, the steady increase in corticosterone concentration continued well beyond point t60, possibly reaching the peak of response at point t120 (*Figure 1*).

Finally, analysis of total corticosterone secretion (AUC_G) revealed a similar pattern. Indeed, differences in the AUC_G were primarily induced by the stress associated with the bleeding procedure rather than by the exposure to the restraint stress ("bleeding technique": F(1,16)=53.091, p<0.0001; "stress exposure": F(1,16)=1.449, p=0.2462; "stress exposure"×"bleeding technique": F(1,16)=2.348, p=0.1450). In particular, both ROS-CTRL and ROS-ARS mice showed significantly increased AUC_G response compared to TAI-CTRL and TAI-ARS mice respectively (*Figure 2*).

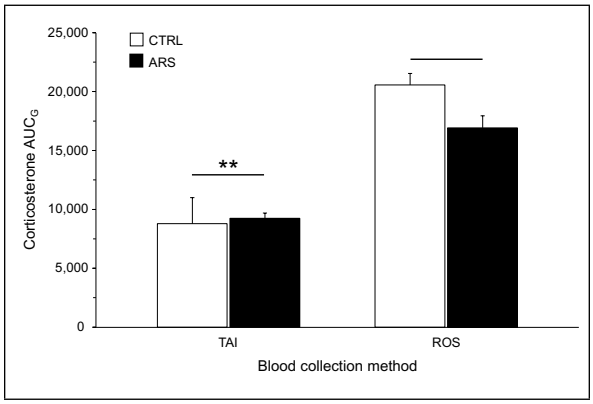

Figure 2
Plasma corticosterone area under the curve with respect to ground (AUC_G; expressed in arbitrary units) in mice bled from either the tail (TAI group) or the retro-orbital sinus (ROS group) and either returned, after baseline sampling, to the home-cage (CTRL group) or exposed to an acute restraint stress (ARS group); **p≤0.01, main effect of the bleeding technique (TAI *vs* ROS; n=10 per group).

DISCUSSION

Here we compared plasma corticosterone concentrations in mice serially bled by using the retro-orbital sinus puncture or the tail incision, either exposed, after baseline sampling, to an acute restraint stress or left undisturbed in their home-cages. We found that the differential increase in corticosterone concentrations was primarily induced by the stress associated with the two bleeding procedures rather than by the exposure to the 25-min restraint stress.

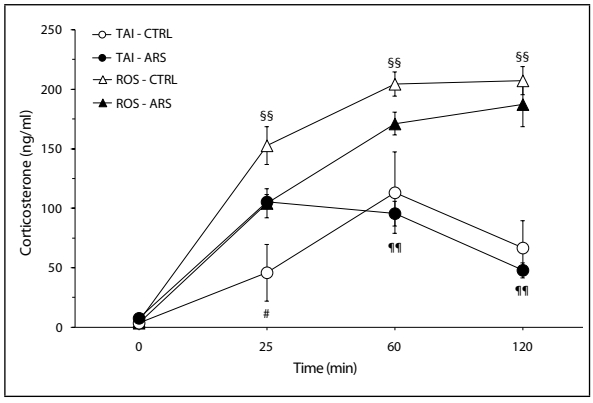

Figure 1
Plasma corticosterone concentrations (ng/ml) at different time points (at baseline and after 25, 60 and 120 min) in mice bled from either the tail (TAI group) or the retro-orbital sinus (ROS group) and either returned, after baseline sampling, to the home-cage (CTRL group) or exposed to an acute restraint stress (ARS group); §§p≤0.01, TAI-CTRL *vs* ROS-CTRL; ¶¶p≤0.01, TAI-ARS *vs* ROS-ARS; #p≤0.05, TAI-CTRL *vs* TAI-ARS (n=5 per group).

In terms of corticosterone peak concentrations, the impact of the two sampling methods was profoundly different, with concentrations in mice bled by retro-orbital sinus puncture almost doubling those of mice bled by tail incision. The marked response observed in mice bled by the retro-orbital sinus could be ascribed to the necessity of firmly restraining the subject for the duration of the blood sampling at each time point and/or to the greater invasiveness of the technique. This interpretation is supported by the absence of a decline in corticosterone concentrations following the ROS procedure even after 120 minutes from the first sampling. However, this procedure is traditionally considered to only cause transient distress and, therefore, the application of a topic ophthalmic anesthetic is usually considered sufficient. By contrast, in rats, because of the presence of a venous plexus rather than a sinus, the use of general anesthesia is widely recommended.

Although general anesthesia is effective to avoid exposure to pain and distress potentially associated with blood sampling procedures, it does not prevent the elevation of plasma corticosterone concentrations. For example, Vachon and Moreau [21] found that (i) corticosterone increased significantly, with a peak at 30 min, in both anesthetized non-cannulated and non-anesthetized jugular-cannulated rats after repeated blood sampling over 2 h, (ii) corticosterone was significantly lower at the 60 and 120 min time points in awake cannulated rats compared with rats undergoing repeated isoflurane anesthesia. Furthermore, mice subjected to both retro-orbital sinus puncture during isoflurane anesthesia and to isoflurane anesthesia alone (no puncture of the si-

nus) showed higher plasma corticosterone concentrations compared to tail incision and tail snip, both performed by means of a restraint device [22]. Finally, Kim and co-authors [3] found that tail snip and retro-orbital sinus puncture in anesthetized mice were associated with higher basal plasma corticosterone levels compared to tail snip in non-anesthetized mice (up to a 20-fold increase). Hence, this elevation was related to the intraperitoneal injection of the anesthetic (ketamine/xylazine) rather than to the bleeding procedure itself. In the present study, we did not find any difference between sampling methods at baseline, likely because the retro-orbital sinus puncture was not performed under general anesthesia.

Mice bled from the tail and mice bled from the retro-orbital sinus showed a different pattern of response over time. While the latter exhibited a steady increase in corticosterone concentrations, which possibly reached the peak 120 min after the baseline, the former showed peak concentrations either at 25 min (mice exposed to restraint stress) or at 60 min (control mice not exposed to restraint stress) time points, followed by a steady decline 120 min after baseline in both groups. In this respect, it should be noted that during bleeding by tail incision, the animal was free to move on the cage's lid. However, transportation of the cage to the adjacent room [4], removal from the home-cage at each sampling point and/or the transient pain associated with the tail incision appeared to have been sufficient to alter secretory patterns of circulating stress hormones, thus inducing a corticosterone response also in mice not exposed to the restraint procedure. Indeed, the two groups of mice bled by tail incision reached similar corticosterone peak concentrations, although they differed regarding time-to-peak, as detailed above.

Based on our results, repeated blood sampling by tail incision does not appear to be a stress-free procedure as previously suggested [6, 12]. These authors found that corticosterone concentration following serial blood collection was either only slightly increased [12] or not increased at all [6] above the baseline of the first sample. However, this conclusion was based on samples collected 80, 100, 120, 150, 180 and 240 min [12] and 24 and 48 h [6] after baseline sampling, when a potential procedure-induced corticosterone elevation might have already returned to baseline. On the contrary, in the present study, we monitored the stress response at an earlier stage, collecting samples 25, 60 and 120 min after baseline sampling. This may have allowed us to evidence an early corticosterone elevation (within 60 min) in control mice (i.e., not exposed to restraint stress) bled by the tail incision method.

Similar results to those achieved in the present study, in terms of both peak concentrations and time course, were obtained by Kim and co-authors [3] using the tail snip method. Specifically, they found that repeated collections (30, 60, 90, 120 min) in freely moving mice induced a significant 10-fold increase in plasma corticosterone after 30 min, which remained at similar levels for the duration of the study, with only a slight decrease at 90 and 120 min. They also found a similar 10-fold increase in corticosterone levels in mice briefly restrained (for less than 2 min) during the bleeding procedure when a second sample was collected 120 min after baseline [3]. Interestingly, Harikrishnan and co-authors [23] found reduced nest building activity in the home-cage, reduced activity in the open field test and increased anxiety in the elevated plus maze test both in anesthetized mice following retro-orbital sinus puncture and in mice bled by tail vein incision while contained in a restraint device.

Notwithstanding the reasons for the more pronounced and prolonged peak in mice bled from the retro-orbital sinus, the exposure to the 25-min restraint stress (immediately after baseline sampling) had no additive effect on the corticosterone response. We believe that the extremely stressful nature of the ROS procedure itself leads to a ceiling effect, thus preventing the detection of a further elevation in corticosterone following ARS exposure. Nevertheless, we cannot exclude that increasing the sample size would allow to detect additional differences between groups. By contrast, in mice bled from the tail, acute stress exposure produced an anticipation of the corticosterone peak from 60 min to 25 min after baseline, that is immediately after the end of the restraint procedure. Based on these data, we conclude that (i) the restraint stress procedure should not be combined with the retro-orbital sinus method as it does not produce any additive effect on the stress response, (ii) in line with the principle of refinement of animal use in laboratory research, combined use of tail incision and restraint stress procedures should be avoided unless it is necessary, for reasons related to the experiment scopes, to anticipate the peak of response (from 60 to 25 min after baseline sampling).

Apparently minor methodological differences in bleeding methods (e.g., using a needle to puncture the tail vein *vs* using a blade to make a tail incision; containing the animal in a restraint device *vs* letting the animal free to move) can have a substantial impact on corticosterone concentrations. The type of material employed for blood collection tubes can also have an impact since hormones tend to adhere to plastic surfaces, potentially distorting subsequent quantitative evaluation (see [24] for a review with historical elements comparing glass and plastic tubes). Hence, results obtained in the present and in the above-mentioned studies strongly support the necessity for accurate reporting of bleeding methods in publications to allow for experimental reproducibility, interpretation of comparative reports and intra-/inter-laboratory variability [25].

Considering strain- and sex-related differences in stress responses, future studies should explore whether our findings extend to females as well as to other strains. Additionally, to capture the full complexity of the stress response, different stressors and multiple physiological outcomes should be incorporated.

CONCLUSIONS

In conclusion, our results support the adoption of the tail incision method, particularly for use in behavioral studies where use of general anesthesia and/or the implantation of indwelling cannulae is not desir-

able. Moreover, we believe that bleeding mice from the retro-orbital sinus should be avoided or performed only under general anesthesia, not only in rats but also in mice.

However, our results also evidenced that the tail incision method is not a stress-free procedure as previously suggested [12, 6]. In particular, this study demonstrates that repeated blood sampling by tail incision in control mice has a considerable impact on the animals' stress response, which should be carefully considered in studies examining the effects of stress and/or assessing behavioral phenotypes potentially affected by stress [23]. Further, as corticosterone levels do not remain low after repeated collections via tail incision in unrestrained, freely moving mice, we conclude that this sampling method can also be used for the study of the acute stress response without the necessity of exposing the animals to a restraint stress procedure. Our findings highlight not only the potential confounding effects of widely used experimental techniques on stress-related endpoints but also their relevance in the context of refinement. By acknowledging and mitigating these effects, animal welfare will be improved while enhancing the reliability of stress-related measurements.

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Authors' contributions

Conceptualization: CM, FF, FZ; formal analysis: MB, FZ; funding acquisition: FZ; investigation: CC, FF; supervision: FZ; visualization: MB; writing – original draft: FZ; writing – review and editing: CM, FF.

Conflict of interest statement

The Authors have no relevant financial or non-financial interests to disclose.

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