Blood Sampling from the Tail Vein, in Comparison with Two Other Techniques, Causes Less Stress to Mice

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Summary
It is important to use the optimal method for repeated blood sampling to ensure minimal stress to mice, and also to provide better pharmacokinetic and pharmacodynamic data. The aim of the present study was to compare the impact of blood sampling methods on corticosterone and adrenocorticotropic hormone (ACTH) levels in mice.
Hsdwin:NMRI mice were divided into four sampling groups: control group (I), vena facialis (II), tail vein (III) and saphenous vein (IV). The first blood samples, obtained from vena facialis, tail or saphenous vein of conscious mice, were taken at time point 0. The second blood sample was taken by decapitation from groups II-IV with isoflurane anaesthesia at time point 20 min. The control group animals were anesthetized and decapitated at 20 min time point. Corticosterone levels in plasma were analyzed at time point 0 and 20 min, and ACTH at time point 20 min.
Saphenous bled mice, in comparison with vena facialis and tail vein sampled mice, indicated statistically significant greater ($P < 0.05$) level of corticosterone at sampling point (0 min). Rising levels of corticosterone in all groups differed statistically ($P < 0.05$) from the control group level, indicating that all tested bleeding methods were stressful to the experimental animals. However, the tail vein bleeding method stressed statistically significantly ($P < 0.05$) less in comparison with vena facialis and saphenous vein bleeding.
At time point 20 min, only saphenous vein bled mice showed statistically significant greater ($P < 0.05$) blood levels of ACTH compared to tail vein bled mice.
Conditions in sampling and rising levels of corticosterone and/or ACTH level did not show direct correlation.
In conclusion the results suggest that the tail bleeding method accomplished least stress to mice and next less vena facialis bleeding. Blood collection technique from the saphenous vein was the most stressful to the experimental animals.

Introduction
The optimal method for collecting blood is important to accomplish minimal stress to mice according to the principles of Replacement, Reduction and Refinement (*Russell, 1957a*), and also to provide better pharmacokinetic and pharmacodynamic data. During blood collection procedures, stress may result from pain, handling and restraint (*Hoff, 2000*). A relatively easy way to obtain blood from a mouse is to puncture the area behind the hinges of the jawbones. This vena facialis blood collection technique has been suggested as a humane and painless tool to draw blood from laboratory mice (*Golde et al., 2005*). It allows for the maximum allowable sample volume with minimal trauma to the animal. This technique can be performed on anesthetized or conscious animals.

Tail vein sampling is a quick and simple method for blood sampling in the mouse (*Bazare et al., 1981*). But requires the animals to be warmed in order to dilate the blood vessel prior to taking the sample.

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This may be stressful and thus affect the experimental data. Sampling from the lateral saphenous vein is a relatively quick method of obtaining blood samples from mice (*Hem et al., 1998*), which should be restrained either manually or using a restraint tube. This can cause stress to the animal and therefore the duration of restraint should be minimized.

Mice have a complex system to adapt to various stressors. This system is collectively called the stress system, and its main components are the hypothalamic-pituitary-adrenocortical (HPA) and the symphathetic-adrenomedullary (SA) systems. In many species, including rodents, corticosterone is the principal glucocorticoid involved in regulation of fuel metabolism, immune reactions, and stress responses (*Hendrich & Bullock, 2004*). Adrenocorticotropic hormone (ACTH) is secreted from the anterior pituitary in response to corticotropin-releasing hormone from the hypothalamus. Corticotropin-releasing hormone is secreted in response to many types of stress. Plasma concentrations and ACTH, causing corticosterone secretion in rodents, and the resulting corticosterone are linked to the experience of stress, and can therefore be used as a stress marker (*Seasholtz, 2000*). However, in previous studies has been indicated that values of corticosterone and ACTH can differ significantly from study to study in rodents (*Tuli et al., 1995a; Tuli et al., 1995b; Tuli et al., 1995c; Shipp & Woodward, 1998; Vachon & Moreau, 2001; Balcome et al., 2004; Gomez et al., 2004; Abelson et al., 2005; Vahl et al., 2005; Altholtz et al., 2006*).

Some of the discrepancy might be explained by the methods of blood collection. Corticosterone in the blood is mostly unbound and can usually be demonstrated after few minutes (*Kugler et al., 1988*). Together with its short half-life, this makes plasma corticosterone a useful parameter for measurement of acute stress. The peak response to corticosterone and ACTH in rodent blood has been reported to be 15-60 min after an acute stress (*Kim et al., 1998; Stagg et al., 2001; Vachon & Moreau, 2001; Vahl et al., 2005; Altholtz et al., 2006*). Not many studies exist in which different blood-taking techniques on plasma levels of stress hormones have been compared in mice.

The aim of the present study was to compare plasma corticosterone and ACTH levels in post-stress mouse blood samples obtained from vena facialis, tail and saphenous vein.

Materials and Methods

The study was performed during the morning hours between 7:00 and 9:00 a.m., when plasma stress hormone levels are expected to be low (*Hendrich & Bullock, 2004*). Four different groups of mice (Hsdwin:NMRI, specific pathogen-free, female, 27.6±1.7 g, age 9 weeks, Harlan Netherlands) were included in the experiment; control group (I), vena facialis (II), tail vein (III) and saphenous vein (IV), 10 mice in each group and 5 animals per cage. To avoid possible hormone level differences caused by daily hormonal rhythm between groups, all groups were evenly distributed to the 2 hour sampling period by varying the sampling order. The study protocol was reviewed and approved by the Animal Care and Use Committee of the State Provincial Office of Southern Finland approval number ESLH-2007-06438/Ym-23.

The animals were housed in a conventional animal room (temperature 21±3 ºC, 12/12 h light/dark cycle). During the experiment the mice were housed in Makrolon 3 cages (Bayer MaterialScience AG, Leverkusen, Germany) that contained rounded plastic mouse houses. They were fed by RM1 diet, batch no 5652 (SDS; Special Diet Services, Witham, Essex, UK) and tap water in bottles was available *ad libitum*. In order to better visualize veins, dilation was accomplished by warming the animals in groups III and IV under a warming lamp for approximately 5 minutes prior to venipuncture. For the same reason, cheek (II) and the tarsal joint area (IV) were slightly shaved prior to blood sampling. The blood sampling was filmed by DVD-camera confirming the same handling of animals during the sampling. In order to minimize differences between samplings the same experienced person were taken samples.

The first blood samples were taken at time point 0, except from the control group. The second blood sample was taken from all animals at time point 20 min by decapitation, which was expected to be least stressful. The purpose of the 20 minute waiting period (II-IV) was to allow the blood corticosterone and ACTH levels to rise before terminal sampling. The purpose of the determination of corticosterone and ACTH levels in the control group was to get information of the baseline level of the stress hormones.

Blood samples (100 µl for 40 µl of plasma) for plasma separation were collected into Capiject® T-MLHG tubes (Terumo Medical Products, Somerset, NJ, USA). Blood samples at time point 0 min were collected from vena facialis (cheek) (II), tail vein (III) and saphenous (femoral) vein (IV) from conscious animals. Blood samples (ca. 500 µl for 200 µl of plasma) from all groups were taken using decapitation under isoflurane anesthesia (isoflurane 4.5% / oxygen 500 cc/min) at time point 20 min. The plasma was separated from whole blood by centrifugation (10 min, 2870 × g) and frozen at -20 ºC within one hour from sampling. The samples were stored at -70 °C until analyzed.

Corticosterone and ACTH levels in plasma were analyzed by corticosterone enzyme immunometric assay (EIA) kit (Immunodiagnostic Systems Limited, Boldon, UK) and ACTH enzyme linked immunosorbent assay (ELISA) kit (BioSupply UK Limited, Bradford, UK) according to the manufacturer´s instructions using Victor^2 1420 multilabel counter plate reader for photometry (Perkin Elmer Wallac, Turku, Finland). Detection limit for corticosterone was 0.55 ng/ml and for ACTH 0.23 ng/ml. The corticosterone and ACTH levels between different groups were reviewed statistically by one-way ANOVA. All statistics were considered significant if P-value was less than 0.05. The independent variables “conditions in sampling”, “rising levels of corticosterone” and “ACTH levels” were estimated by calculation of Pearson's correlation coefficients (*r*).

Results

Results of the corticosterone and ACTH analysis are presented in Figure 1 and 2, respectively. Rising levels of corticosterone in all groups differed statistically ($P < 0.05$; $N = 10$ / group) from the control group level, indicating that all tested bleeding methods were stressful to the experimental animals. Also the tail vein bleeding method differed from vena facialis and saphenous vein bleeding ($P < 0.05$). Saphenous bled mice, in comparison with vena facialis and tail vein sampled mice, indicated statistically significant greater ($P < 0.05$) level of corticosterone at sampling point 0 min.

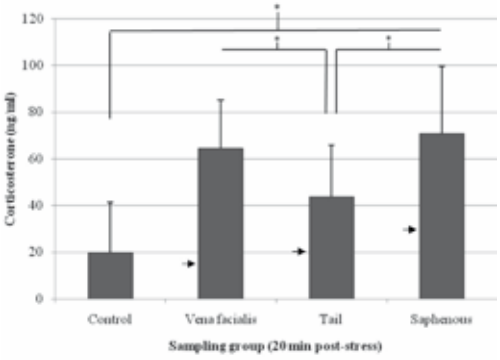

Figure 1. Corticosterone analysis. Rising levels of corticosterone 20 min post-stress. Error bars = SD. * = Statistical difference between sampling groups ($P < 0.05$). Arrows indicate corticosterone level at the initial sampling point (0 min).

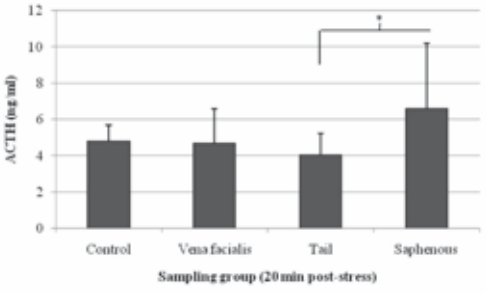

Figure. 2. ACTH analysis. ACTH levels 20 min post-stress. Error bars = SD. * = Statistical difference between sampling groups ($P < 0.05$).

The only difference detected in the ACTH levels is that the saphenous vein method differs statistically ($P < 0.05$; $N = 10$ / group) from the tail vein bleeding method. At time point 20 min, only saphenous vein bled mice showed increased (16%) blood levels of ACTH over the control group level. However, no statistical differences were found between control group and the tested methods.

Some general information of the sampling is presented in Table 1. Only minor differences can be found in the time of warming between test groups at time point 0 min. A difference can also be found in the number of vein punctures among groups at time point 0 min. The average number of punctures is the lowest in the tail vein puncture group. For the vena facialis puncture group the average number of punctures is 14% higher, and 28% higher for the saphenous vein puncture group. At time point 20 min, the time of anaesthesia is also comparable in all other groups except the saphenous vein puncture group, which is about a minute longer than in the three other groups. However, conditions in sampling and rising levels of corticosterone and/or ACTH level did not show direct correlation. A statistically significant association (Pearson´s correlation coefficient and P-value) was only shown between average time in anaesthesia and ACTH level ($r = -0.802$, $P = 0.01$) for the vena facialis bleeding group.

Discussion

The study reported here compares the impact of blood sampling methods that are currently accepted and widely used, on corticosterone and ACTH levels in Hsdwin:NMRI mice. Plasma corticosterone and ACTH in post-stress mouse blood samples obtained from vena facialis, tail and saphenous vein were measured. During blood sampling in rodents, stress may result from pain, routine handling, unfamiliar environment and restrain (Gärtner et al., 1980; Tuli et al., 1995; Tuli et al., 1995b; Tuli et al., 1995c; Shipp & Woodward, 1998; Li et al., 2000; Vachon & Moreau, 2001; Rogers et al., 2002; Balcombe et al., 2004; Alholtz et al., 2006).

Differences in blood corticosterone levels between the experimental groups at sampling point 0 min. were observed. Corticosterone levels were 15.8±19.3 ng/ml (avg±SD) (vena facialis), 20.9±21.6 ng/ml (tail) and 30.0±16.8 ng/ml (saphenous). The reason for this difference can be speculated that vena facialis bleeding doesn't require warming prior to sampling, and for this reason the method is faster to perform than the two other methods in the present study. Then again tail vein bleeding and saphenous vein bleeding require warming in order to better visualize veins prior to sampling. Thus the total handling time is longer and adequate for the blood corticosterone concentrations to rise from the base line level already during the first sampling, causing a greater corticosterone level than in vena facialis bleeding at blood samples taken at time point 0 min. In comparison with the previous studies, plasma baseline corticosterone levels coincide with those previously observed in mice (Tanoue et al., 2004; Weber et al., 2006).

Based on the levels of corticosterone in the control group without initial sampling stress at 20 min time point, the present study suggests that mice anesthetized by isoflurane / oxygen prior to decapitation did not affect to stress response. The measured level of corticosterone in the control group corresponded with levels observed with low-level stressed mice (Shipp & Woodward, 1998). Controversially the

Table 1. Conditions in sampling. - = Not done. * = Statistically significant association ($P < 0.05$)

Group	Control	Vena facialis	Tail	Saphenous
Average warming time (min) ± SD	-	-	4.6 ± 0.7	4.4 ± 1.2
Average number of vein puncture ± SD	-	1.8 ± 1.1	1.6 ± 0.7	2.0 ± 1.0
Average time in anaesthesia (min) ± SD	2.4 ± 2.2	2.8 ± 0.4*	2.6 ± 0.7	3.6 ± 2.1

corticosterone levels of the control group were significantly lower than levels reported with isoflurane anesthetized mice (Shipp & Woodward, 1998). In our experiment, the anesthesia chosen was acknowledged by authorities. Further, Altholtz et al., 2006 observed, based on reduction in stress, that isoflurane as opposed to CO2 is the better choice when anesthesia is needed for serial blood collection in rats. The corticosterone levels in the control group indicate that isoflurane / oxygen anesthesia prior to decapitation is a humane method and causes less stress during euthanasia of mice.

In the present study, corticosterone levels in the different sampling groups were 64.8±18.2 ng/ml (avg±SD), 80.3±21.5 ng/ml and 86.3±31.0 ng/ml in tail, vena facialis and saphenous vein bled mice 20 min post-stress, respectively. According to the results of the present study, tail vein bleeding seems to inflict the least blood corticosterone concentration compared to the control group level. However, there was statistically significant difference ($P < 0.05$) between tail vein bled and control group mice, suggesting that all blood sampling techniques tested are stressful to the mice. Both vena facialis and saphenous vein bleeding produce a greater corticosterone concentration than tail vein bleeding. Furthermore saphenous vein method seemed to cause an even greater corticosterone level than vena facialis method, but this difference wasn't statistically significant. Conditions used for sampling and rising levels of corticosterone and/or ACTH level did not show direct correlation. Hence, these results suggest that tail vein bleeding itself is less stressful to the mouse than the vena facialis and saphenous vein bleeding tested in this study.

Balcombe et al. (2004) concluded that common methods of blood sampling were associated with increased levels of corticosterone in rats and mice. Tuli et al. (1995a) demonstrated that corticosterone levels were significantly higher in mice immediately after the completion of tail bleeding than in control mice suggesting that tail bleeding in mice was stressful. Abatan et al., 2008 compared saphenous venipuncture and modified tail-clip blood collection in mice. They reported that the two methods produced similar corticosterone responses. In addition, the effects of saphenous venipuncture appeared to be dependent on the handler's technical expertise. In the present study samples from all three puncture sites were taken by the same experienced person in order to minimize differences between samplings.

ACTH level was only measured at 20 min time point. The reason for this was the limited volume of plasma for analyses, and thus corticosterone was chosen to indicate rising level of the stress hormone (samplings at time point 0 and 20 min). The ACTH concentrations measured here were 4.1±1.2 ng/ml (avg±SD), 4.4±2.3 ng/ml and 6.2±4.0 ng/ml in tail, vena facialis and saphenous vein sampled mice 20 min post-stress, respectively and shows statistically significant difference ($P < 0.05$) between saphenous vein against tail vein bleeding. The slight discrepancy between corticosterone and ACTH levels in mice was also observed in a previous study (Jezova et al., 2004). Comparison of the present study results with existing information on ACTH levels in mice is difficult, because the methods used for the analysis differ between the studies. Statistically significant differences ($P < 0.05$) in ACTH analysis between saphenous vein against tail vein bleeding groups, support the findings from the corticosterone analysis. However, the reason for the experimental groups' similarity in ACTH concentrations might also be a result of a too long waiting period (20 min) prior to the terminal sampling. In this case the rise in ACTH levels, due to handling and restraint in blood sampling, have already had time to return to control levels. Another problem is the time required for anaesthesia before decapitation. The handling of the animal might cause ACTH levels to rise again. A quicker terminal sampling, for example lethal pentobarbital sodium injection or no anesthesia, would have been better for ACTH determination (Vahl et al., 2005).

In conclusion, the results obtained from the corticosterone and ACTH analyses suggest the tail vein puncture method to be less stressful to the animal than the vena facialis or saphenous vein puncture

method. The information gathered in this study proposes the tail vein bleeding to be used rather than vena facialis or saphenous vein bleeding. This is a refinement suggestion according to the principal of the 3R's, and will provide better wellbeing of laboratory mice.

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