

The Effect of Automated Blood Sampling on Corticosterone Levels, Body Weight and Daily Food Intake in Permanently Catheterized Male BALB/c Mice

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Abstract. *Background/aim:* Automated blood sampling has the benefit of sampling without human intervention, thus minimizing the associated stress response. Since this technique has not been thoroughly investigated in mice, the present study was designed to evaluate this technology in mice. *Materials and Methods:* Male catheterized BALB/c mice were subjected to automated blood sampling, fecal sampling and daily recording of body weight and food intake for three days post-surgery. Corticosterone levels in blood and feces were investigated as biomarkers of stress. *Results:* Plasma corticosterone levels were elevated, and the circadian rhythm was disrupted as reflected in both plasma and feces. The body weight and daily food intake declined for the first two days post-surgery and increased at day three. *Discussion:* These results demonstrate that surgery and subsequent automated blood sampling induce a stress response for up to three days post-surgery, and it is concluded that further refinement of this technique is essential.

Repeated blood sampling from mice is a common feature of many studies. Manual blood sampling is associated with a stress response in the animal, which is a welfare concern and may be a source of experimental bias. Development of refined techniques to obtain series of blood samples from individual mice is valuable in order to improve animal welfare and reduce variation in the experimental results.

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Permanent catheterization and automated blood sampling (ABS) has been investigated in rats (1-4). In mice, permanent catheterization has been performed (5-7), but ABS in mice is technically more difficult and less well-described in the literature. The Accusampler[®] (Dilab, Lund, Sweden) is designed to collect blood samples automatically, thus with potentially less disturbance of the animal compared to manual blood sampling. Volumes from 5 to 50 µl (plus a waste volume of approximately 5 µl) can be sampled. Thus several blood samples can be withdrawn from the same animal, resulting in fewer animals being required for certain types of studies. From an ethical point of view, this method may be an improvement, as it potentially concurs with two of the 'Three Rs' by reducing the number of animals required in some studies and by refining the method of repeated blood sampling in studies where serial blood samples from individual animals are needed. However, the technique requires surgical implantation of a vascular catheter during anesthesia, and the stress associated with catheterization has only been infrequently studied in mice (8). Thus, further investigation and refinement of this technique is essential.

The present study aimed to investigate how mice recover from catheterization and to what extent the ABS procedure in itself causes stress to the animals. To assess the stress response associated with ABS, corticosterone levels in blood and fecal corticosterone metabolites (FCM) were quantified, and body weight (BW), as well as the daily food intake (DFI), were recorded for three days post-surgery.

A study performed by Macleod and Shapiro (9) showed that permanently catheterized mice had lower levels of circulating corticosterone compared to unstressed mice and experimentally stressed mice. The catheterized mice were sampled for blood between two and 40 days post-surgery but not the first two days post-surgery. A previous study at our laboratory (8) showed that surgical placement of an arterial catheter induces a significant stress response in the first 24

hours post-surgery, as judged by elevated plasma corticosterone and reduced BW. The stress response after catheterization is a combined effect of anesthesia, surgery and blood sampling and cannot be separated into its constituents, which is why a longer study period after catheterization was important to investigate. Previously, it has been shown that stress relating to anesthesia and surgery passes within 24-48 h post-surgery (10, 11). Consequently, elevated corticosterone and FCM levels and disrupted corticosterone and FCM rhythmicity for more than two days post-surgery would, in the present study, be interpreted as being an effect of the ABS method. It was hypothesized that if mice subjected to ABS were stressed by the procedure, circulating corticosterone levels would be increased and the normal rhythmic pattern of corticosterone secretion and FCM would be altered during the first three days post-surgery. It was furthermore hypothesized that the BW and DFI would decline during this period, if mice were stressed by ABS.

Materials and Methods

This experiment was approved by The Animal Experiments Inspectorate under the Danish Ministry of Justice (license number: 2009/561-1687). The animals were at all times handled by trained personnel. In total, 34 male BALB/cJBomTac mice, 6 to 8 weeks old (Taconic, Ry, Denmark), were randomly divided into an experimental group with 10 mice weighing 24.8 ± 0.46 g (mean \pm SEM), and a control group with 24 mice weighing 25.2 ± 0.52 g (mean \pm SEM).

Housing conditions. Single housing was necessary to avoid catheter damage. To habituate the mice to single housing, they were singly housed from the day of arrival in disposable cages (Innovive Inc., San Diego, USA) in an individually ventilated cage system. After arriving at the facility, the mice were acclimatized for seven days before experimentation. Wooden chips (Tapvei Oy., Korteinen, Finland) were used as bedding material. Bite bricks (Tapvet®, Korteinen, Finland) and Enviro-dri® nesting materials (Shepherd Specialty papers, Quakertown, Pennsylvania, USA) were used for environmental enrichment. Before surgery, the mice were given a cardboard roll (Mini fun tubes; Lillico, Brogaarden, Gentofte, Denmark) as a hide. A diurnal rhythm was maintained with a 12:12 hour light-dark cycle, with artificial light from 06:30. Room temperature was kept at $21 \pm 2^\circ\text{C}$, air humidity at $55\% \pm 10\%$, and the air was exchanged approximately 20 times/hour. After catheterization, the mice in the experimental group were housed in polycarbonate cages (Dilab, Lund, Sweden), designed to be used with the Accusampler®, and transferred to a designated room with calm environmental conditions similar to those described above. They were provided with food pellets (Altromin 1319; Brogaarden, Gentofte, Denmark) and acidified tap water *ad lib*.

Surgery. Surgery was commenced at 9:30 day 0. Anesthesia was induced with 5% isoflurane (Forene®; Abbot Scandinavia; Stockholm, Sweden) conveyed in 100% oxygen, and maintained at 2.5% isoflurane in 100% oxygen. Mice were provided anesthesia by spontaneous breathing through an anesthetic face mask (AgnTho's, Lidingö, Sweden). The mice of the experimental group were catheterized in the common carotid artery with tunneling of the catheter subcutaneously to the mid scapular region of the neck.

Table I. Outline of project. Surgery was performed at 9:30 day 0. Feces were sampled from day 0 to day +3. Blood was sampled from day +1 to day +3. Body weight and food intake was recorded each morning. After the final sample at 8:00 day +3, the mice were euthanized.

| | Day 0 | Day +1 | Day +2 | Day +3 |
|-----------------------------|-------|--------|--------|--------------------|
| Body weight and food intake | 08:00 | 08:00 | 08:00 | 08:00 |
| Buprenorphine | 08:00 | 08:00 | 08:00 | |
| | 16:00 | 16:00 | 16:00 | |
| Catheterization | 09:30 | | | |
| Fecal sampling | 08:00 | 08:00 | 08:00 | 08:00 |
| | 14:00 | 14:00 | 14:00 | |
| | 20:00 | 20:00 | 20:00 | |
| Blood sampling | | 08:00 | 08:00 | 08:00 |
| | | 14:00 | 14:00 | |
| | | 20:00 | 20:00 | |
| | | 02:00 | 02:00 | |
| Euthanization | | | | After final sample |

After surgery, the mice were immediately connected to the Accusampler®, which flushed the catheter every 20 minutes with 20 μl of 25 IU/ml heparinized saline to maintain patency.

The mice were given pre-emptive analgesia consisting of 1 mg/kg BW buprenorphine (Temgesic; Schering-Plough Europe, Brussels, Belgium) mixed in a commercially available nut paste (Nutella®; Ferrero, Pino Torinese, Italy), given for voluntary ingestion at 8:00 the day of surgery and then twice daily until euthanization. Buprenorphine tablets (0.2 mg) were crushed to a fine powder before mixing with 1g Nutella to ensure an even concentration of 0.2 mg buprenorphine/g Nutella. The dose of buprenorphine was based on recommended doses (12, 13), as well as our own experience (14, 15). To habituate them to the flavor, the mice were offered pure Nutella in the morning and in the evening for two days prior to surgery. This has been shown to promote the voluntary ingestion of the Nutella-buprenorphine mixture as pre-emptive analgesia (14). The mice were handled daily to habituate them to the experimenter and to minimize possible stress response related to fecal sampling.

Sampling. Blood samples of 25 μl (plus a waste volume of 5 μl) were obtained automatically every six hours from 8:00 the first day post-surgery until 8:00 the third day post-surgery, resulting in nine blood samples per animal (Table I). The heparinized blood samples were centrifuged to isolate plasma and stored at -21°C until analysis. Plasma corticosterone levels were quantified with an enzyme-linked immunosorbent assay (ELISA) kit (EIA-4164; DRG Diagnostics, Marburg, Germany) in accordance with the manufacturer's instructions.

Feces were sampled three times daily at 8:00, 14:00 and 20:00 for three days post-surgery. All fecal pellets were collected and stored at -21°C until extraction. FCM levels were quantified by ELISA as described previously (8).

BW and DFI were recorded each morning throughout the experiment. After the final sample, the mice were euthanized with 10 mg pentobarbital/kg BW (Veterinærapoteket, University of Copenhagen, Denmark) injected through the catheter (16).

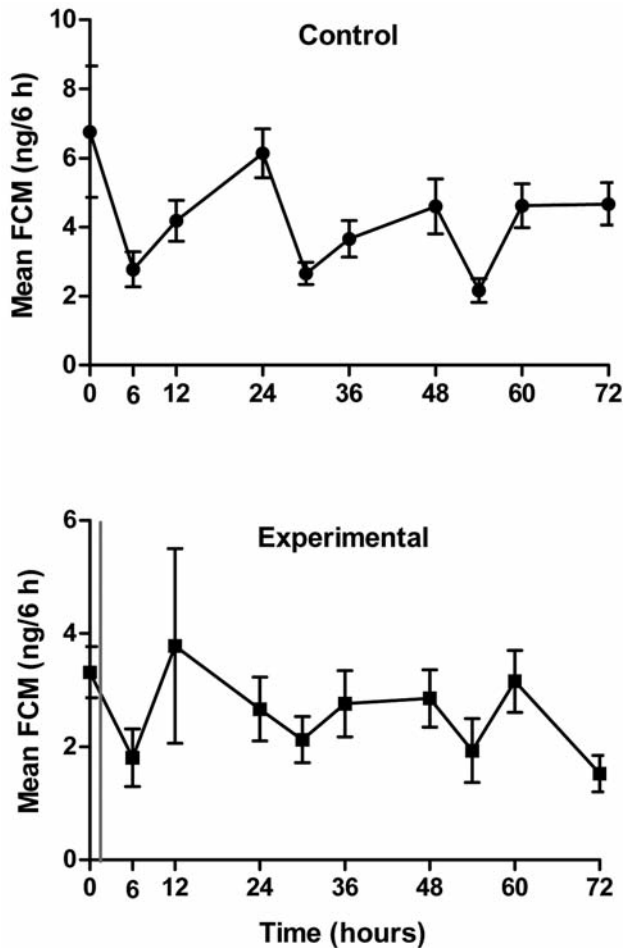


Figure 1. Fecal corticosterone metabolite (FCM) level and circadian rhythm. Values represent the mean \pm SEM for 24 control mice and 10 experimental mice sampled three times daily at 8:00, 14:00 and 20:00 for 72 hours from day 0 until day 3 after surgery. Surgery was performed at 9:30 day 0 (grey line).

Control animals. To determine the normal FCM levels and the associated normal circadian rhythm, feces were sampled three times daily (8:00, 14:00, 20:00) for three days from non-operated control animals. BW and DFI were recorded each morning throughout the experiment. The mice were housed under similar conditions as described for the experimental animals before surgery. The FCM levels, BW changes and DFI from these mice served as a reference for the results from the operated animals. The plasma corticosterone values from the experimental mice were compared to values described in literature (16, 17).

Data treatment. Data are presented as the mean \pm SEM. Where relevant, the data were analyzed in SPSS Statistics 20 (IBM, Armonk, NY, USA) and analyzed for normality using Shapiro-Wilk tests. As a few data sets within both the experimental and the control groups did not exhibit a Gaussian distribution, non-parametric tests were used to investigate the differences within and between groups. The Mann-Whitney *U*-test was used to compare the overall

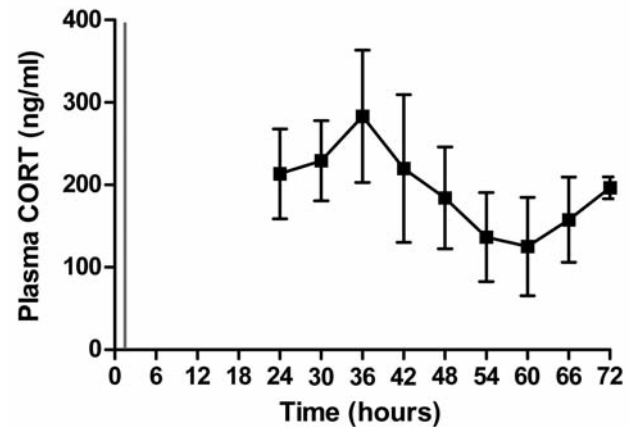


Figure 2. Plasma corticosterone (CORT) level. Values represent the mean \pm SEM for seven experimental mice sampled four times daily at 8:00, 14:00, 20:00 and 02:00 for 48 hours from one day after surgery. Surgery was performed at 9:30 on day 0 (grey line).

difference between the experimental group and the control group. Statistics are presented as a *U*-value and an asymptotic significance (two-tailed) where *p*-values <0.05 were interpreted as being significant. A Friedman test was used to test the difference across days for individual groups with a Wilcoxon signed-rank *post-hoc* test. Statistics are presented as a chi-square value, $\chi^2_{(df)}$, where *df* are the degrees of freedom, and an asymptotic significance where *p*-values <0.05 were interpreted as being significant.

Results

Figure 1 illustrates the FCM levels in 24 control mice and 10 experimental mice, sampled three times daily at 8.00, 14.00 and 20.00 for 72 hours post-surgery. The FCM level in the control mice followed a diurnal rhythm, which was maintained between 2-7 ng/6 h (mean total FCM) and peaked at 24, 48 and 72 hours post-surgery, corresponding to 8.00 each morning. The mean FCM values of the experimental mice were maintained between 1.5-4.0 ng/6 h (mean total FCM), with no clear apparent diurnal rhythm from 12 hours after surgery.

Mean plasma corticosterone values of seven experimental mice are shown in Figure 2. The mice were sampled automatically four times daily at 8.00, 14.00, 20.00 and 02.00 from 8.00 the day after surgery and for 48 hours. Levels ranged from 100-300 ng/ml (mean total corticosterone) and no apparent circadian rhythm was evident during the whole sampling period.

Figure 3 illustrates the change in BW (mean g/day) and DFI (mean g/day) during four days from day 0 to day 3, where surgery was performed at 9.30 day 0. It is evident that the experimental mice had a significantly reduced BW all three days post-surgery compared to the control group

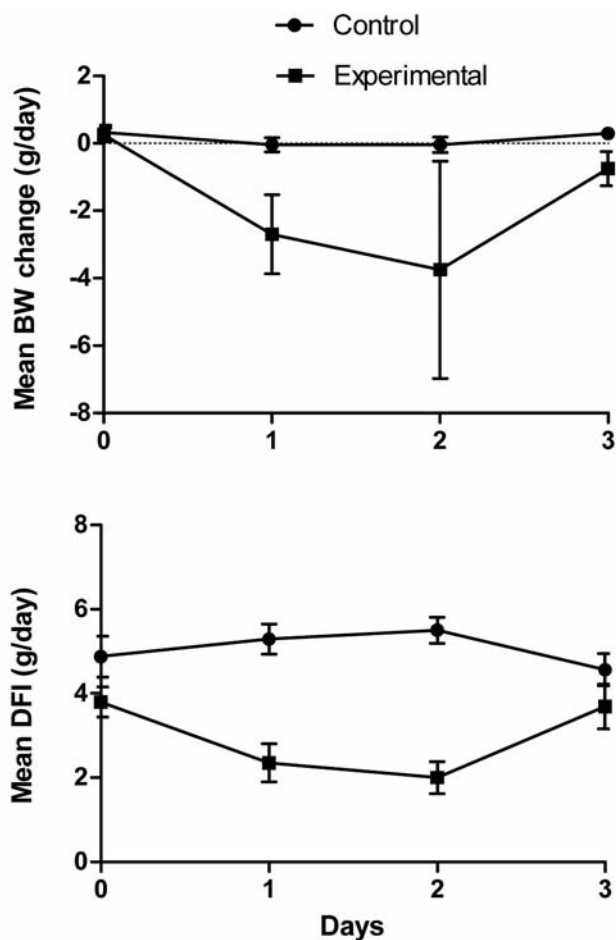


Figure 3. Body weight (BW) change and daily food intake (DFI). Values represent the mean \pm SEM for 24 control mice and 10 experimental mice sampled for four days at 8:00 from day 0 until day 3. Surgery was performed at 9:30 on day 0.

($U=1351.5$; $p=0.009$). There was no significant difference between days within either the control group ($\chi^2_{(3)}=2.131$, $p=0.546$) or the experimental group ($\chi^2_{(3)}=4.469$, $p=0.215$).

The DFI was, on average, significantly higher in the control mice compared to the experimental mice ($U=658.0$; $p<0.001$). There was no significant difference between days within the control group ($\chi^2_{(3)}=6.147$, $p=0.105$). Within the experimental group, there was a significant difference between days ($\chi^2_{(4)}=12.907$, $p=0.012$), with a significant decrease in the mean DFI from day 0 to day 1 ($p=0.021$) and day 2 ($p=0.026$), and a significant increase from day 2 to day 3 ($p=0.011$).

Discussion

Elevated FCM levels and a disrupted pulsatility were anticipated in relation to surgery, since anesthesia and surgery are stressors known to affect endocrine homeostasis



Figure 4. The Accusampler μ ® system. The mouse is fitted with a harness which is connected to a flexible spiral tether and a mobile swivel at the top of the cage. The catheter is coupled to a dual line tube which runs inside the tether and connects to the swivel. A balance arm maintains the correct tension on the catheter and the swivel allows free movement in all directions.

by increasing circulating corticosterone levels and disturbing its rhythmic pattern (10, 17, 18). However, the FCM levels of the experimental mice ranged within 1.5-4.0 ng/6 h and thus were not elevated compared to those of the control animals. On the other hand, from approximately 12 hours after initiation of the experiment (at 20:00 day 0), and throughout the remainder of the experiment, the circadian rhythm in these animals was disrupted. The lag time for circulating corticosterone to be excreted in feces has been shown to be 8-10 hours in mice (19, 20), explaining the time gap from surgery to the rhythmic disruption of FCM levels. In rats, it has been shown that FCM is a valuable tool for measuring severe and chronic stress (1, 21). However, a recent study on mice concluded that fecal corticosterone quantification was unsuitable for assessing postoperative stress after permanent catheterization of the carotid artery (8), at least during the first 24 hours after surgery. In the present study, FCM was quantified for three days following surgery, but not even in this period, were elevated FCM levels observed. The results of the present study indicate that measures of FCM levels may be a less suitable method for assessing postoperative stress in mice compared to rats, and that the stress response associated with catheterization of mice can be detected in feces only as a disruption of the normal diurnal pattern. This is most likely due to a different excretory pattern of corticosteroids in mice compared to rats, which, however, still remains to be fully understood.

Seven out of ten mice were successfully sampled for blood. Three mice were excluded from blood sampling due to technical failure or problems with catheter patency.

Compared to the literature, the observed plasma corticosterone levels, which ranged from 100-300 ng/ml (mean total corticosterone) without an apparent circadian rhythm, are considered elevated. Normally, circulating corticosterone levels express a clear rhythmic pattern that peaks in late afternoon of 150-200 ng/ml and then declines during the night to levels below 50 ng/ml until around 10:00, after which the levels increase again (16, 17). The elevated plasma corticosterone values and the disturbed rhythmic pattern in the present study indicate some stress in the animals, which can be partly explained by the anesthesia and surgery, which in this setup involved the first 24 hours of blood sampling (10, 11). The levels of the final 24 hours of blood sampling are, therefore, interpreted as mainly being an effect of the ABS method itself. In this period, the corticosterone levels declined towards normal levels and approached a normal circadian rhythm.

As mentioned, it has been shown that permanently catheterized mice have lower levels of circulating corticosterone compared to unstressed mice and experimentally stressed mice, between two and 40 days post-surgery (9). Although the experimental setup in that study was different from the one in the present study, it is possible that the plasma corticosterone concentration might normalize after a few days post-surgery, and that permanently catheterized mice are likely to be less stressed than mice subjected to repeated manual blood sampling.

BW decreased significantly after surgery in the experimental group compared to the control group. There was no significant difference between days within the groups, however, the BW of the experimental animals declined the first two days post-surgery and then increased again towards normal levels. This relates very closely to the DFI, which was also significantly lower in the experimental animals compared to the control mice. The DFI of the experimental animals decreased for up to two days post-surgery, then increased and approached normal levels. If a longer study period had been used, an increase in BW would likely have been observable as a result of the increased food intake. It is therefore concluded that the effect of surgery is visible on BW and DFI for up to three days post-surgery.

The analgesic protocol has previously been validated by our laboratory as sufficient for catheterization of mice and rats (4, 8, 22); the stress response is believed to be a consequence of the method rather than of pain related to the surgery. However, buprenorphine is known to inhibit food intake (23, 24), which could have an impact on DFI and BW in the present study. However, the mice still expressed some stress associated with the procedure as demonstrated by the corticosteroid results, and even after termination of the analgesic treatment, FCM, plasma corticosterone, BW and DFI were altered, most likely as a consequence of the ABS method.

ABS should theoretically cause minimal disturbance of the animal at the time of sampling. The necessary housing conditions associated with use of the Accusampler[®] system require an accurate setup that might have an impact on the animal (Figure 4). The catheter is connected to a dual line tube (Dilab, Lund Sweden) that runs inside a flexible spiral tether, which protects the catheter. The mouse is fitted with a harness to support the catheter at the exit in the back. At the top of the cage, the dual line tube is connected to a balance arm that follows the mouse's movements. A swivel on the balance arm can turn around its own axis, allowing the mouse to move freely around the entire cage. The distance from the cage to the sampler must be set correctly, as this can influence the properties of the balance arm because of strain on tubings. Too tight a balance arm will pull the mouse at the wound in the neck, and the mouse will consequently avoid moving around. Conversely, too loose a balance arm will weigh upon the mouse, creating unnecessary discomfort. This system most likely influences circulating glucocorticoid levels the first few days after connection to the system.

In this study, the mice were transferred immediately after surgery to the Accusampler[®] cage system and allowed to regain consciousness in the new surroundings. As the animals were still anesthetized during transfer, this is not expected to influence the stress response. However, cage change and the restraining devices associated with the ABS method (tether and harness) most likely influence glucocorticoid levels. Based on the results, however, this stress response seems to normalize after approximately three days and it is reasonable to speculate that a few days of habituation to the system is sufficient. Furthermore, a recovery period between surgery and sampling is advised in order to minimize the stress response associated with anesthesia and surgery and hence reduce the effect on the scientific results.

In conclusion, the Accusampler[®] is designed to sample very small volumes of blood, making it ideal for repeated blood sampling from small laboratory rodents. With adequate skills in surgery and technical management of the system, it is possible to sample blood automatically, potentially improving repeated blood sampling techniques in mice. However, the results in the present investigation indicate that the method is stressful to mice for at least up to three days after catheterization. More research is necessary in order to enable us to reduce this stress response, and hence refine the ABS technology.

Competing Interests

The Accusampler[®] was donated by Dilab, Lund, Sweden with the purpose of testing the applicability of the Accusampler[®] for automated blood sampling in mice. However, the Authors decline any conflicts of interests that could have biased the judgment or interpretation of the results in the present report.

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