

High Plasma Corticosterone Levels Persist During Frequent Automatic Blood Sampling in Rats

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Abstract. *Corticosterone levels in blood may be used as a marker of stress in rodents, provided that the blood sampling procedure itself is non-stressful. Automated blood sampling equipment (Accusampler[®]) allows blood sampling without any interference with the animal and might be useful as a tool for an on-line measurement of stress markers in blood. However, the impact of the blood sampling itself on the corticosterone levels in blood is unknown. The present study was designed to evaluate whether the frequency of blood sampling influences the plasma corticosterone levels in male and female rats. During anaesthesia, a catheter was placed in the jugular vein and attached to an Accusampler[®]. Blood samples (200 µl) were withdrawn with a high (24 samples) or low frequency (3 samples) during a six-hour period immediately after the catheter insertion. The corticosterone levels in the plasma were quantified with ELISA. The corticosterone levels persisted at high post-operation concentrations when blood was collected frequently, while the levels steadily declined significantly during low-frequency sampling. The corticosterone levels were higher in female than in male rats, but the curves were similar. The present study elucidates the importance of considering the frequency of blood withdrawal during automated blood sampling. This parameter may have an impact on the experimental results when using blood corticosterone levels as a stress marker, but also during any *in vivo* study where blood is collected, since high corticosterone levels may affect the normal physiology of the animals.*

Stress in laboratory animals is an obstructive circumstance in most experimental conditions, since stressors significantly alter the normal physiology and metabolism and, thereby, increase variation within and between individual animals.

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This makes stress a major source of experimental error (1, 2). In addition, persisting stress is followed by several adverse effects such as immunosuppression, which results in an increased susceptibility to infectious diseases (3, 4). Stress must, therefore, be considered not only as a confounding variable in experimental results, but also as a major cause for suffering in laboratory animals, and to strive for reduction of stressful conditions is essential during normal husbandry, as well as during and after experimental procedures.

In a stressful situation, adrenal glucocorticoid steroids are produced and they are considered the main mediators for the immunosuppressive status during persistent stress (5). The most important glucocorticoid in rodents is probably corticosterone, which increases rapidly in blood during a stressful situation and can serve as a marker for adrenal function (6, 7). In order to use corticosterone as a marker for stress, it is vital that the sampling method is not in itself stressful. Corticosterone in blood may be used as a stress marker if blood is collected under non-stressful conditions, such as automated sampling without handling, since manual blood sampling entails a stressful situation for the animal. The Accusampler[®] (DiLab, Lund, Sweden) is a computerised, fully automatic blood sampler that enables administration of drugs into the blood circulation of conscious rats, as well as collection of blood samples, without any human interaction except for the surgical insertion of a catheter in the jugular vein. The ability to withdraw blood without disturbing the animal by handling makes the Accusampler[®] a very applicable tool for physiological and biological studies in laboratory animals *in vivo*. In addition, it should also make it a suitable tool for measuring blood levels of corticosterone, using this molecule as a stress marker. A previous study in our laboratory investigated the correlation between serum corticosterone and corticosterone metabolites in faeces in rats connected to an Accusampler[®], over a period of 94 h after surgery (8). Apart from this study, little has been published regarding the impact of automated blood sampling on stress perception in the animal. One advantage

of the Accusampler® is that it allows repeated blood sampling at frequent intervals without any loss of body fluid, since the withdrawn blood volume is immediately replaced by saline. This allows studies of biological agents in blood at several time-points during a shorter period, and corticosterone should be no exception.

The surgical procedure for catheterising animals is associated with stress. Apparently surgery causes painful tissue damage, which could lead to stress and elevated corticosterone levels in the post-operative phase (9). In addition, anaesthetic agents during surgery, including isoflurane that was used in the present study, are well-known stress factors (5, 6). In order to minimise experimental errors when using laboratory animals that have gone through surgery, it is of great importance to better understand the physiological stress responses that occur during the postoperative phase. Automated blood sampling with the Accusampler® should be appropriate as a tool for a thorough investigation of this phenomenon. However, the impact of the automated blood sampling itself on the corticosterone levels requires much more evaluation before the Accusampler® can be considered as a non-stressful tool for stress marker measurement.

The aim of this study was to evaluate the impact of sample frequency during automated blood sampling on the corticosterone levels in blood, by studying corticosterone levels from rats where blood samples were collected at a high frequency compared with rats where blood samples were obtained less frequently. The study also aimed to evaluate whether gender differences would influence the corticosterone levels in blood during frequent blood sampling.

Materials and Methods

Animals. All animal experiments in this study were approved by the Animal Ethics Committee in Uppsala, Sweden. Twelve male and four female outbred Sprague-Dawley rats (B&K, Sollentuna, Sweden) were used in the study. The male rats weighed between 350 and 500 g before surgery, while the female rats weighed 200-400 g. The rats were housed in Makrolon type III cages one week before use and kept in animal rooms with standard animal house conditions: diurnal rhythm was regulated with a 12 h light:12 h dark cycle with light on from 6 a.m. to 6 p.m.; temperature was kept at $21 \pm 1^\circ\text{C}$; relative humidity was 30-60%; and cages were cleaned twice a week. Aspen chips (Finn Tapvei, Kortteinen, Finland) were used as bedding material. The animals had free access to food pellets (R36 Laktamin, Stockholm, Sweden) and tap water at all times.

Surgical procedure. Prior to surgery, the rats were treated with a subcutaneous injection of 0.05 mg/kg buprenorphine (Temgesic, Schering Plough Europe, Brussels, Belgium), to ensure adequate post-operative analgesia. The rats were anaesthetised using 5% isoflurane (Forene, Abbot Scandinavia, Kista, Sweden) delivered in 100% oxygen. Once anaesthetised, the rats were attached to a Simtec anaesthetic mask for spontaneous respiration and the level of isoflurane was maintained to ensure adequate anaesthesia. A vein catheter (DiLab) filled with heparinised saline was inserted

into the jugular vein with the tip close to the entrance of the right atrium of the heart. The catheter was secured in the vein by two sutures and led subcutaneously through a DiLab Dacron button attached to the skin in the dorsal region of the neck. The catheter was led further through a spiral and connected to the Accusampler®. The experimental set-up is shown in Figure 1.

Blood sampling. All experiments were performed during six hours immediately after surgery. In the high-frequency blood sampling experiments (five male and four female rats), blood samples were collected every 15 minutes commencing during the recovery phase, before the rats regained consciousness. The volume of each blood sample was 200 μl . Immediately after each sample, the Accusampler® injected 200 μl saline into the rat, in order to preserve the total blood volume and thereby maintain circulatory functions. In the low-frequency control experiments (seven male rats), blood samples were collected on three occasions only: during the recovery phase, after two hours and finally after six hours. The volume of each blood sample in the control experiments was 150 μl and, as in the frequent sampling experiments, the withdrawn blood volume was immediately replaced by saline. All experiments were ended by decapitation of the rats.

Corticosterone analysis. The blood samples were collected in heparinised tubes and stored overnight at 4°C , after which they were centrifuged to remove blood cells and obtain plasma. The plasma concentration of corticosterone was quantified using enzyme-linked immunosorbent assay (ELISA). The OCTIGEN ELISA (Immuno-Diagnostics Systems Ltd., Tyne and Wear, UK) and Correlate ELISA (Assay Design Inc., Ann Arbor, MI, USA) kits were used for quantification of corticosterone, according to the manufacturers' manuals. The intra-assay coefficient of variation was approximately 2.1% and the inter-assay coefficient was 6.3%. **Statistics.** For studying the corticosterone trends, determination of the slope of the curves was performed by linear regression using the GraphPad Prism 4.0 software. To compare corticosterone levels between male and female rats after frequent blood sampling, the general linear model with repeated measures (SPSS ver. 11.5) was used. *P*-values < 0.05 were considered significant.

Results

When blood samples were collected every 15 minutes, the plasma corticosterone level remained high during the six-hour sampling period without any sign of decline (Figure 2). When blood samples were collected at only three time-points during the six-hour sample period, the first sample was approximately at the same level as for the male rats in the high frequency experiments, but throughout the experimental period the corticosterone level declined significantly (Figure 2).

The corticosterone levels were significantly higher in female rats than in male rats during frequent blood sampling, but the curves were similar in shape (Figure 3).

Discussion

The Accusampler® has been developed as a device for automated blood sampling and administration of substances

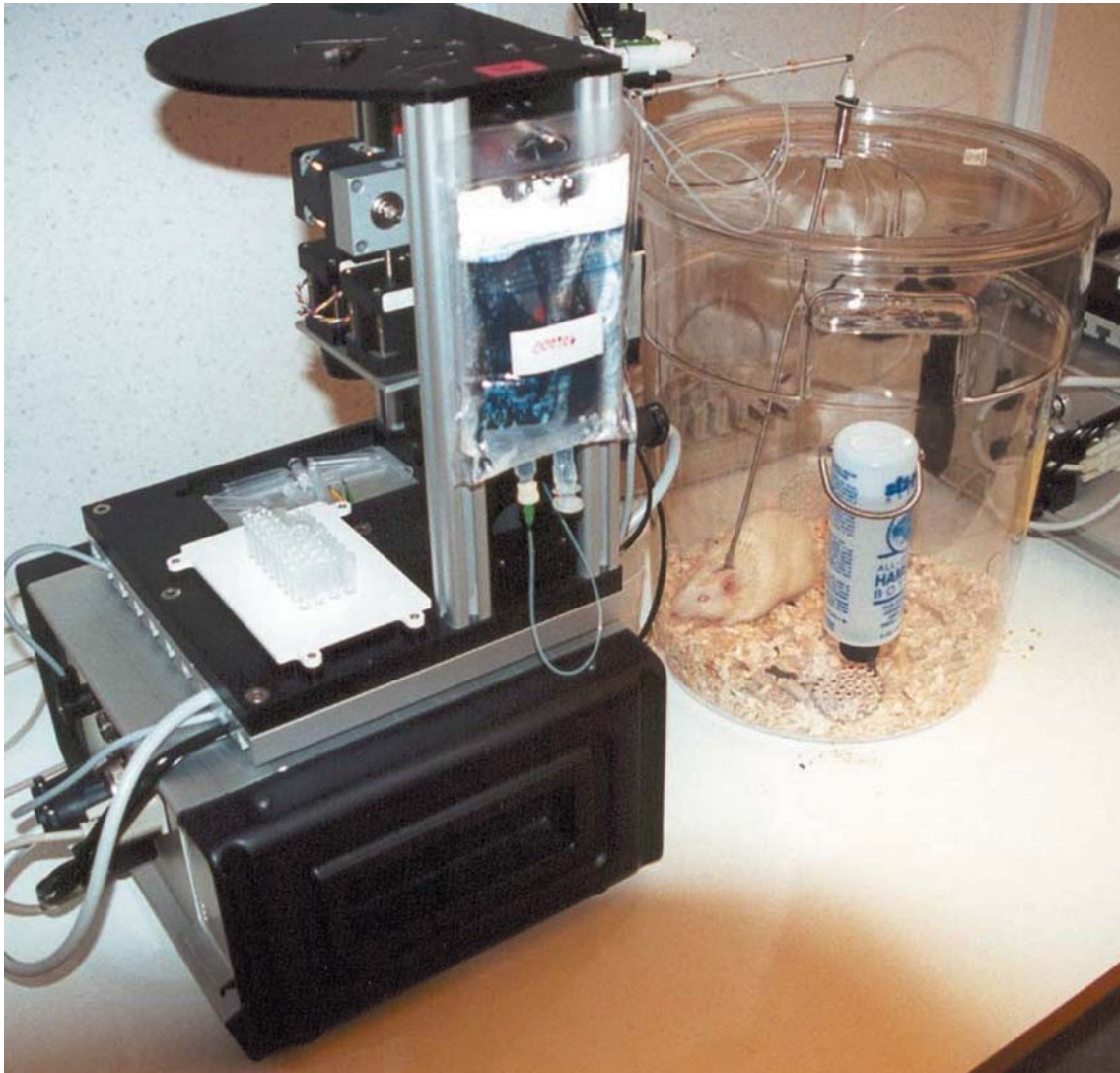


Figure 1. *Experimental set-up with the Accusampler®. Photograph is printed with kind permission from DiLab.*

without any interference with the animals. This is essential in many studies, since handling and manual blood sampling is associated with stress, which impairs the experimental results and the well-being of the animal. However, the effects of the automated blood sampling on the animals must be further evaluated to avoid biased interpretation of data from the samples obtained.

It is evident from the present data that the frequency of the blood sampling significantly affected the results in the experimental set-up described in this study. When only three samples were drawn during the study period, the plasma corticosterone levels showed a significant gradual decline, as described by Royo and co-workers (8). The

persistence of high corticosterone levels during frequent blood sampling every 15 minutes probably reflects an influence of the frequent blood sampling itself, rather than a prolonged stressful condition after surgery. An explanation for this could be that each blood withdrawal procedure is experienced as stressful by the animal, but a more reasonable explanation is that the high, persistent corticosterone levels are due to an adaptation of circulatory functions. Corticosterone is an important hormone in the regulation of circulatory functions, such as maintenance of vascular tone (10, 11), endothelial integrity (12) and distribution of body fluids (13). In addition, it is well known that the corticosterone levels in the circulation increase

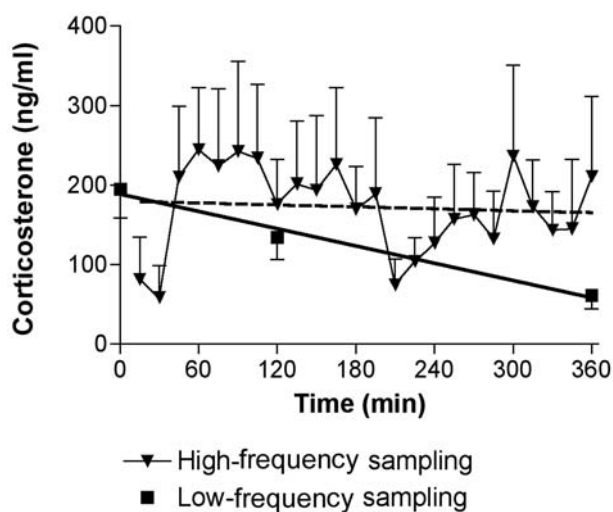


Figure 2. Corticosterone levels in blood of male rats during six hours after surgery, as obtained by high-frequency or low-frequency blood sampling. The corticosterone levels during low-frequency sampling significantly declined in the observed period (unbroken line), as determined by linear regression (slope = -0.36 ± 0.10 ; $F_{(1,18)} = 11.92$; p -value < 0.003). Corticosterone levels did not decline (broken line) during high-frequency sampling (slope showed no deviation from zero; p -value 0.78).

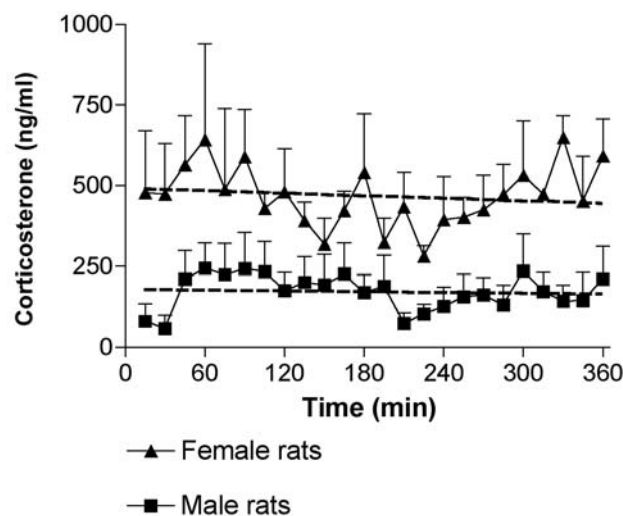


Figure 3. Corticosterone levels in blood in male and female rats during the first six hours after surgery, as obtained after high-frequency blood sampling. The corticosterone levels were significantly higher in female than in male rats at all time-points except time = 150 min, as determined by general linear model with repeated measures ($F_{(1)} = 11.12$; p -value < 0.05). The corticosterone levels did not decline during the observed period (broken line), either for male or female rats, as determined by linear regression (slope showed no deviation from zero; p -values were 0.78 and 0.64 for male and female rats, respectively).

during haemorrhagic shock in rats (14-16). Thus, frequent blood sampling may induce an elevated corticosterone release as a response to the blood withdrawal, similar to the response after a controlled haemorrhage. The Accusampler[®] replaces the withdrawn blood volume with saline in order to maintain the fluid balance and circulatory functions. Apparently, fluid replacement with only saline is not sufficient when blood is withdrawn at such a high frequency as used in the present study.

Although it is evident that the high-frequency blood sampling during the six-hour period caused high and persistent corticosterone levels in rats, it appears that the frequency and volume used in the present experimental set-up is just on the border of what could be described as haemorrhagic blood withdrawal. This is supported by the observation shown in Figure 3, where it is clear that both male and female corticosterone levels tend to decline in the time-interval between 100 and 200 minutes, which is what would be expected during a normal recovery, as seen in the control group (Figure 2). Due to the continued blood sampling, however, the corticosterone levels are again elevated and persist at a high level. Irrespective of the exact limit at which the blood sampling becomes haemorrhagic, the evident effect of high-frequency blood sampling on corticosterone levels demonstrates that the frequency of blood sampling is crucial,

not only for the use of blood corticosterone as a stress marker, but for all studies that attempt to investigate biological processes *in vivo* by using automated blood sampling, since an elevated corticosterone level affects the normal physiology and homeostasis of the animal.

The data presented in this study clearly demonstrate that the corticosterone levels were significantly higher in female than in male rats. This confirms previous studies, suggesting that the gender differences are related to sex steroids such as estrogens (17) or androgens (18). The reason for studying both male and female rats in the present study was to evaluate whether the hormonal differences would influence the pattern of blood corticosterone during frequent blood sampling, which was not the case. The level was higher in females than in males, but the graphs were very similar.

In conclusion, the present data clearly demonstrated that the sampling frequency during automated blood sampling directly influences the results with regard to the corticosterone levels in blood. High-frequency blood sampling is associated with persistent high levels of corticosterone, while low-frequency sampling is associated with declining levels of corticosterone. The corticosterone levels during high-frequency sampling are higher in female rats than in male rats, but the non-declining pattern is equal in both sexes. Thus, the

frequency of blood sampling should always be taken into consideration when planning and designing animal experiments using automated blood sampling.

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