Evaluation of stress response using saliva sample in adult male mouse

Summary of the Doctoral Thesis

Masakatsu Nohara

(Supervised by Professor Hiromi Amao)

Doctoral Course of Applied Life Science

Graduate School of Veterinary Medical and Life Science

Nippon Veterinary and Life Science University

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Plasma and serum samples are generally acceptable for detection of various physiologically active substances, while drawing blood could be stress for human and animals. In case of collecting repeated blood samples from mouse, using saphenous and lateral tail vein are recommended, and these sites yield 5% of circulating blood volume from saphenous veins and 0.1–0.15 ml from lateral tail vein, and the detection of the substances by any assay in this amount of blood sample is limited. Blood sampling by decapitation or cardiac puncture is employed as collection to whole blood, and it obtained approximately 1 ml of whole blood and modestly 0.5 ml of plasma. This volume is enough to measure multiple substances by an immunoassay, and in this case, a large number of mice are needed as a result of the sacrifice of animals at each time point of any responses to experimental treatments. In addition to this problem, it is impossible to investigate multiple time points, before and after responses to the treatment in the mouse of the same individual.

Recently, saliva is recognized as low-invasive sample for detection of physiologically active substances instead of plasma or serum. In this study for evaluation of stress response in the adult male mouse, we measured salivary corticosterone levels and salivary amylase activity, because glucocorticoids and salivary amylase have been known as biomarkers of stress response. However, in the small experimental animals such as mice and rats, few studies have been examined evaluation of salivary corticosterone and salivary amylase activity as a stress biomarker.

In order to clarify the whether salivary corticosterone is available to evaluate stress response in mice, 1. we confirmed that the corticosterone was detected in mouse saliva and that the glucocorticoid transferred from blood to saliva after administration of exogenous cortisol (chapter 2), 2. compared salivary secretion in mouse treated different anesthetic agents, and corticosterone levels between plasma and saliva in response to restraint stress, and evaluated saliva secretion in mouse treated with anticancer drug (chapter 3), and 3. examined adequate recovery periods from anesthesia for saliva collection between pre-stress and post-stress and also examine salivary corticosterone and amylase activity in mice (chapter 4).

1. ***Measurement of glucocorticoids levels using mouse saliva sample (chapter 2)***

We confirmed that corticosterone in saliva was detected by enzyme immunoassay (EIA) and that glucocorticoids transferred from blood to saliva in mice treated with 2.0 mg/kg via intraperitoneal (ip) injection of cortisol. Cortisol is the glucocorticoid produced principally in humans but not in rodents. Saliva secretion was enhanced by pilocarpine hydrochloride (0.5 mg/kg ip). Obtained saliva and plasma samples used for measurement of cortisol levels by EIA.

Salivary corticosterone was detected by EIA and the cortisol was detected in plasma and saliva in mice as a result of cortisol injection. Conversely, in the mice treated with vehicle, the cortisol was not detected in saliva but it existed at very low levels in plasma. In this study, anti-cortisol showing 2% cross-reaction with corticosterone was used for EIA. The reason why cortisol detected in vehicle control group, might account for the cross-reactivity of anti-cortisol.

Results of this study revealed that the salivary corticosterone was detected by EIA, and the results indicate that the origin of cortisol detected in mouse saliva is considered as exogenous corticosteroid, and the cortisol could be transferred from blood to saliva via salivary glands. From these results, we concluded that the glucocorticoids in saliva might reflect to the levels of its blood circulation.

2. ***Investigation of anesthetic agent and stress conditions for evaluation of adrenal function using saliva sample of mouse (chapter 3)***

We compared the effects of two different types of anesthetic agents (mixed anesthetic agents and pentobarbital) on salivary secretion. Mixed anesthetic agents consisting of medetomidine, midazolam and butorphanol are regarded as anesthetic agent instead of pentobarbital generally used in animal experiments. In this study, the mixed anesthetic agents were composed of medetomidine hydrochloride (0.3 mg/kg ip), midazolam (6.0 mg/kg ip) and butorphanol tartrate (7.5 mg/kg ip), and pentobarbital sodium was treated at a dosage of 40 mg/kg intraperitoneally injection. We also compared corticosterone levels between plasma and saliva in response to restraint stress with or without the mixed anesthesia. Mice were immobilized for 60

minutes by restrainer. Furthermore, we examined effect of anticancer drug (cyclophosphamide, 50 mg/kg ip) on salivary secretion and on salivary corticosterone levels.

Salivary corticosterone levels and volume of saliva were not significant differences between the mixed anesthesia group and pentobarbital anesthesia group. However, salivary protein levels in the mixed anesthesia group were significantly lower than the levels in the pentobarbital anesthesia group. The plasma corticosterone levels of restraint (60 min) group were significantly higher than the levels of the non-stress control group with or without anesthesia. The salivary corticosterone levels of restraint group were also significantly higher than the levels of the control group. Cyclophosphamide treated group did not show significant increase of salivary corticosterone levels compared to the vehicle control group. Moreover, volume of saliva secreted and salivary protein levels were not shown significant differences between control and cyclophosphamide groups.

Results of this study indicate that the mixed anesthetic agents are recommended agent for saliva sampling. The salivary corticosterone levels are significantly increased as a result of 60 minutes immobilization. The results suggest that salivary corticosterone levels reflect changes in plasma corticosterone levels caused by restraint stress in the mouse. Moreover, cyclophosphamide (50 mg/kg ip) does not significantly affect salivary secretion and on salivary corticosterone levels in mouse in the present study.

3. ***Investigation of the adequate recovery period from anesthesia for saliva collection (chapter 4)***

We evaluated the adequate recovery period from anesthesia for saliva collection between pre-stress and post-stress in adult male mouse. In the investigations evaluating for recovery periods from anesthesia, four different time points (1, 3, 5 and 7 days) were set as a recovery periods. Salivary collection was divided into two fractions (0–20 and 20–40 min). Salivary corticosterone was determined by EIA and the amylase activity was measured using a dry chemistry system.

Restraint stress increased significantly corticosterone levels of saliva collected for 40 minutes in all four groups. On the other hand, the statistical evidence of corticosterone increase is more rigorous in a 7-day recovery group ($p < 0.001$) than the others ($p < 0.05$). Moreover, salivary amylase activities of prior fraction were shown significant increase in 3- and 7-day recovery groups by restraint stress, but not in 1- and 5-day recovery groups. Conversely, the activities of posterior fraction were shown significant decrease in 1- and 5-days recovery groups, and the activities in 3- and 7-days recovery group was unchanged by restraint stress.

Results of this study indicate that recovery period from anesthesia for saliva collection in stress experiment is preferable for 7 days in the same individual of mice. Furthermore, to evaluate salivary amylase activity in response to restraint stress using saliva sample, saliva sample in early period of saliva collection would be preferable.

In conclusion, we suggest that the collected saliva is available for EIA of corticosterone and that the salivary corticosterone levels reflect the plasma corticosterone levels, and it will be a useful less-invasive biomarker of physical stress in mice. Moreover, to evaluate restraint stress by salivary corticosterone level and amylase activity in mice under anesthesia, adequate recovery period from anesthesia is preferable for a week. Additionally, the present study may contribute to concepts of Reduction and Refinement of the 3Rs in small animal experiments.