Changes in salivary stress markers under experimental xerostomia in the rat

Yoichi Tsuji, Kenji Uchihashi and Yasuo Nishikawa

Department of Physiology, Osaka Dental University, 8-1 Kuzuhahanazono-cho, Hirakata-shi, Osaka 573-1121, Japan

Saliva sampling has the advantages of being easy, stress free and non-invasive, allowing for multiple sampling. We examined how xerostomia that was induced by botulinum toxin A treatment affected the salivary cortisol and amylase levels, and compared the characteristics of these parameters. Twenty male Wistar rats weighing 280–320 g were used in this experiment. After botulinum toxin A treatment, the stress response was determined using salivary cortisol and amylase levels during chorda-stimulation. The amylase level was significantly decreased on day 7 after botulinum toxin A was administered. However, the cortisol level was increased, and the increase was to a lesser extent than that of amylase. Both the salivary cortisol and amylase levels recovered over time, suggesting that they are good indices of xerostomia. Furthermore, it is suggested that amylase is an index of relaxation. (J Osaka Dent Univ 2011; 45: 185–192)

Key words: Xerostomia; Botulinum toxin; Submandibular gland

INTRODUCTION

Physical or psychological stress is an inevitable fact of life. Stress can be defined as a disruption of normal homeostasis.1,2 The physiological response to stress is complex. Under stress, the human body responds physiologically by increased activity of both the hypothalamic-pituitary-adrenal corticomedullary (HPA) axis and the sympatho-adrenal system (SAS).3 The HPA axis is responsible for the synthesis and/or release of three key hormones: corticotropin-releasing hormone, adrenocorticotropic hormone, and a species specific glucocorticoid, either cortisol in humans or corticosterone in rodents.4 The SAS exerts the “fight or flight” response that depends upon the release of the catecholamine neurotransmitter. The sympathetic stress response increases the heart rate, blood pressure, and blood glucose levels in muscles and vital organs to help the body adapt to the increased demand.5 In addition, it has been reported that the serum level of cortisol increases in a stress response.

Based on the knowledge that the stimulus of the psychological stressor precipitates a series of compensatory mechanisms expressed as discernible physiologic changes, increasing attention has focused on identifying and measuring the corresponding biologic indicators.7 Once the perception of threat recedes, the negative feedback mechanisms help restore hormone levels to baseline. However, if the stressors are extreme or chronic, the homeostatic process may become dysregulated and provoke the altered neuroendocrine patterns associated with various psychopathological conditions.8 Considerable evidence has shown a link between neuroendocrine dysregulation and psychopathology, including mood and anxiety disorders.9,10

The centrality of hormonal stress mediators to normal and maladaptive stress responses renders them an attractive means of connecting the stress experience with the individual’s psychobiological response to trauma. However, the intrusiveness and logistical limitations inherent to the pervasive use of blood and urine as sources of peripheral biomarkers have led to growing interest in the use of saliva as an alternative.11 A virtual mirror of the body, saliva can reflect practically the entire spectrum of normal and disease states, including tissue levels of natural substances as well as hormonal and immunologic status.12 The three major salivary glands
and numerous minor glands produce ample amounts (500–1500 mL) of saliva daily.13

For patients, supplying a saliva sample evokes less anxiety than providing a blood sample, and less embarrassment than producing a urine specimen.

Unlike the phlebotomy skills required for blood collection, saliva samples are easily procured. Multiple sampling over the day or over many days can be readily completed in the field or at home, thus increasing the feasibility of doing longitudinal studies.14 Saliva requires minimal manipulation because it does not clot, and raises fewer ethical concerns than more invasive methods. Furthermore, saliva samples can be obtained without difficulty from children15 and individuals with physical or mental handicaps.16 The noninvasive collection of saliva is particularly advantageous when subjects require regular monitoring with repeated sampling.

In summary, saliva is an ideal bio fluid for biomarker discovery and profiling of mental health disorders and is a promising basis for inexpensive, noninvasive, and easy-to-use diagnostic technology.

Various diseases are accompanied by a relative or absolute state of hyposalivation, which causes great discomfort for the patient. This symptom often occurs in neurologic disorders, such as Parkinson’s disease and myasthenia gravis, and particularly in children with cerebral palsy,17 who suffer severe drooling. On the other hand, xerostomia is a condition characterized by decreased salivary flow. There are many causes for hyposalivation, including systemic diseases that accompany aging, and a number of commonly used medications.18 Reduced salivary function results in an unstable oral ecology whereby bacterial levels, salivary flow, and caries susceptibility are altered. These alterations in the oral environment may ultimately result in tooth erosion, increased plaque accumulation, gingival inflammation, and oral moniliasis.19,20 Many elderly experience dry mouth for a variety of reasons.21,22

Interestingly, output from the major salivary glands does not undergo clinically significant decrements in healthy elderly.20

The therapeutic use of muscarinic agents is sometimes avoided because of under lying diseases. The use of artificial saliva may inconvenience patients during talking. Recent clinical research on the gene transfer of aquaporin is still in progress.24,25 Salivary dysfunction is any alteration in the qualitative or quantitative output of saliva caused by an increase or decrease in salivary output.26 The terms hyposalivation and xerostomia are often incorrectly used interchangeably. Hyposalivation refers to a diminished salivary flow, whereas xerostomia refers to a subjective experience of mouth dryness. This is further complicated by the fact that some patients with hyposalivation do not have xerostomia and, conversely, those with xerostomia may have normal salivary flow rates. However, xerostomia is a common symptom associated with salivary gland hyposalivation. Usually when salivary secretion has decreased to half its normal amount, an individual will begin to experience xerostomia.27

Injection of botulinum toxin type A (BoNT) into the salivary glands was one of the first alternatives to traditional treatments for sialorrhea in neurodegenerative diseases.28 Small placebo-controlled and open studies documented its efficacy and safety in patients with Parkinson’s disease,29,30 and amyotrophic lateral sclerosis.31,32 Pharmacological denervation of the salivary glands by intraglandular application of BoNT causes inhibition of acetylcholine release at the neuroglandular junction and produces a distinct reduction in salivary flow. BoNT selectively inhibits the cholinergic components, while the adrenergic innervation is left mainly intact.

We investigated the relationship between xerostomia and stress marker (α-amylase and corticosterone) during experimental xerostomia and stress.

MATERIALS and METHODS

Experimental animals

Approval was obtained from the Animals Research Committee of Osaka Dental University (10–02018), and the experiments were performed according to the Ethical Guidelines of the International Associa-
tion for the Study of Pain. Twenty adult male Wistar rats weighing 280–320 g were used. Experimental animals were divided into two groups, control and BoNT treated rats (Fig. 1). The animals were housed in the animal care center under controlled light and environmental conditions (12/12 h dark/light cycles; 23 ± 1°C; 55% relative humidity). Dry food and water were available ad libitum. The experiment began with one day of familiarization with the box. Both water and food ingestion was recorded, beginning 24 h before each experiments.

**Measurement of water and food ingestion**

Both water and food ingestion were recorded, beginning 24 h before each experiments. The amount of drinking water was weighed 24 h before beginning the experiment. The volume of water ingestion was calculated per 100 g of animal weight per day. The volume of food consumed was calculated by subtracting the number of remaining pellets from the total pellets. The amount of food ingested was calculated per 100 g of animal weight per day. Body weight was measured immediately before each experiment.

**BoNT treatment**

In order to treat them with BoNT, the rats were first anesthetized with thiopental (50 mg/kg, ip). Intraductal injection of BoNT was applied to the submandibular gland via polyethylene tubing (Intramedic PE-10; Becton & Dickinson, Sparks, MD, USA), which was inserted into the oral opening of the submandibular gland duct. The gland was injected with solutions using a micro infusion pump (Model 55–111; Harvard Apparatus Ltd., Edenbridge, UK). The time from beginning of the injection until pressure release was 3 minutes. The bilateral submandibular glands were treated in each experimental rat once with the Botulinum toxin A (BoNT; Sigma-Aldrich Inc.) with each animal receiving a total injection of 20 μL of BoNT (5 U BoNT in 0.1 mL saline). The control rats were treated with the same volume of saline in a similar manner. Experimental rats were divided into two groups, Experiment 1: one dose of BoNT at the beginning (Fig. 2), and Experiment 2: two doses of BoNT on day 6 and 13 after at the first dose of BoNT (Fig. 3). On day 7 and 14 after the first treatment of BoNT or saline, the following analyses were done.

**Measurement of salivary flow rate**

Saliva samples induced by iv administration of 8 mg/kg of pilocarpine hydrochloride (PR) were collected from the submandibular gland through polyethylene tubing (Intramedic PE-10; Becton & Dickinson) inserted into the oral opening of the main ducts. Fluid volume of the collected saliva was then measured.

**Salivary α-amylase assay**

A chemistry calibrator kit (Vitros; Roche, Mannheim, Germany) was used for analyzing the level
of α-amylase in the saliva. The principle of the assay is the AMYL Slide, a dry, multilayered, analytical element that is coated on a polyester support. A 10 mL drop of sample was deposited on the slide and evenly distributed by spreading the surface layer to the underlying layers. The spread layer contained the dyed starch substrate for the reaction. The amylase in the sample catalyzed the hydrolysis of this dyed starch into smaller dyed saccharides. These dyed saccharides diffused into the underlying reagent layer. The reflection density of the dyed saccharides in the reagent layer was measured by reflectance spectro-photometry at 2.3 and 5 min. The change in the reflection density between the two times was proportional to the sample's amylase activity.

Salivary corticosterone assay
Salivary corticosterone was assayed using corticosterone Enzyme Immunoassay Kit (CorrelateEIATM kit, Assay Designs Inc. Ann Arbor, MI, USA).

General design of experiment shows in Fig. 4.

RESULTS

Water ingestion of rats
Water ingestion significantly increased after BoNT treatment compared with same aged control rats. The mean increase in water ingestion was 190% on day 14 (Fig. 5).

Food ingestion of rats
Food ingestion decreased after BoNT treatment compared with control rats of the same age. The mean reduction in food ingestion was 86% on day 14 (Fig. 6).

Ratio of increase body weight
The body weight of the controls was increased 136% compared with the start of the experiment, while the BoNT treated rats only increased by 123%. Body weights in the BoNT treated rats were substantially less (Fig. 7). These results indicate that reduction of salivary secretion may induce oral dysfunction, such as xerostomia.

![Fig. 4 General design of experiment](Image)

![Fig. 5 Differences in ingested drinking water between the animals with BoNT injected in the submandibular glands and those with the saline injected in the contralateral submandibular glands (Mean ± SD).](Image)

![Fig. 6 Differences in ingested food between the animals with BoNT injected in the submandibular glands and those with the saline injected in the contralateral submandibular glands.](Image)
Experiment 1
Salivary flow rate
There was no spontaneous secretion from the submandibular gland. The muscarinic agonist pilocarpine (PR) was infused intravenously in the anesthetized rats. The intravenous injections of PR elicited successively increased secretory responses in an age dependent manner. However, the increase in the salivary flow rate was decreased in the BoNT treated rats, by 66% on day 1, by 58% on day 7 and by 56% on day 14 compared with control rats of the same age (Fig. 8).

Salivary corticosterone level
There was no age dependent alteration in the level of salivary corticosterone in the control rats. There was no difference between the controls and the BoNT treated rats on day 1. However, there was a slight increase of 37% in the level of salivary corticosterone on days 7 and 14 in the BoNT treated rats, compared with control rat of the same age (Fig. 9).

Fig. 7 Differences in body weight between the animals with BoNT injected in the submandibular glands and those with the saline injected in the contralateral submandibular glands.

Fig. 8 Salivary secretion elicited by pilocarpine (8 mg/kg iv) on the controls and rats treated with BoNT.

Fig. 9 Salivary corticosterone elicited by pilocarpine (8 mg/kg iv) on the controls and the rats treated with BoNT.

Fig. 10 Salivary α-amylase elicited by pilocarpine (8 mg/kg iv) on the controls and rats that received a single dose of BoNT.
Salivary α-amylase level
There was a slight increase in the level of salivary α-amylase in control rats in an age dependent manner. There was a significant increase of α-amylase after BoNT treatment to 180% on day 1 in same age control rats. Although α-amylase maintained a high level on day 7, it was decreased compared with day 1, and then decreased gradually to that of the control level by day 14 as same as (Fig. 10).

Experiment 2
Salivary flow rate
Although the addition of BoNT on days 6 and 13 resulted in a further decrease in salivary flow rate on days 7 and 14, there was no significant difference with the rats that received the single BoNT dose (Fig. 11). This salivary flow level was the minimum that occurred with BoNT treatment.

Salivary corticosterone level
The addition of BoNT, there was a further increase in the level of salivary corticosterone on days 7 and 14 in the BoNT treated rats, compared with experiment 1 (Fig. 12).

Salivary α-amylase level
There was a significant increase of α-amylase after the addition of BoNT treatment on day 1. Although α-amylase was decreased on day 7 in case of a single dose of BoNT, it was slightly increased by additional treatment of BoNT. However, this level was decreased to the control level on day 14 (Fig. 13). The results were similar to those of experiment 1.

DISCUSSION
This study attempted to examine the relationships between α-amylase activity and glucocorticoid, which results from stress, during xerostomia. The physiologic effects of steroid hormones are initiated when the enter target cells and bind to steroid receptors, which act as transcriptional activators of steroid-responsive genes. A major fraction of steroids bind to carrier plasma proteins. Protein binding
affects the steroids' availability for the target tissues, and their elimination rate; both of which influence their physiological functions. Free and non-specifically bound steroids are physiologically available, whereas steroids bound to the binding globulins have more limited physiological activity. In the past, although protein-bound steroid hormones were considered inactive, it has been shown that androgens and estrogens play a role in the development and maturation of the reproductive system.\textsuperscript{34}

We found that the physiological measures, including salivary $\alpha$-amylase and glucocorticoid, changed in the experimental group prior to and after xerostomia, in contrast to the control group. Our finding of increased salivary $\alpha$-amylase following psychological stress is consistent with previous studies.\textsuperscript{35} However, the present study failed to show overall perfect correlations between the level of salivary $\alpha$-amylase and corticosterone levels. These correlations were only partially supported. Although a number of previous studies tried to examine a direct relationship between salivary $\alpha$-amylase and other adrenergic markers, they also were not able to demonstrate correlations among them.\textsuperscript{36,37} These findings may suggest that salivary $\alpha$-amylase is stress dependent, with its increase reflecting changes in the autonomic nervous system in general. Additionally, the response over time of salivary $\alpha$-amylase when the participants were under psychological stress was very close to that of the catecholamine, blood pressure, and heart rate.\textsuperscript{37} Increases in salivary $\alpha$-amylase were instantaneous in response to stress and rapidly decreased after stress. These responses implicate salivary $\alpha$-amylase as a very sensitive marker of stress.

The present findings have a few clinical implications. Evaluation of the physiological response to stress will not be limited. Nurses are able to scrutinize the effects of stress, including physiological and psychosocial aspects, from a holistic point of view. This information also provides basic knowledge that is important in understanding the mechanisms of the development of xerostomia that are induced by stress. In addition, the measurement of salivary $\alpha$-amylase offers a way to evaluate the effectiveness of newly developed stress management interventions. In our study, in contrast to basal HPA and SAM (sympatho-adrenal medullary) activities, exposure to stress induced by xerostomia resulted in some corticosterone endocrine-response profiles.

In the BoNT treated gland on day 1, the $\alpha$-amylase response to xerostomia was significantly greater than to corticosterone. There was no difference in the corticosterone response on day 1 compared with that on days 7. $\alpha$-Amylase responses were significantly increased on day 1 in the BoNT treated gland. To date, the attempts to correlate personality traits have focused mainly on cortisol release.

Corticosterone response to the stressor was significantly lower in females than in males, while there was no gender difference in the cortisol response of the low-anxiety group. Gender differences in the amylase response were not observed in either the high- or low-anxiety groups. To date, the attempts to correlate personality traits have focused mainly on corticosterone release.

We measured levels of salivary $\alpha$-amylase and corticosterone. In addition, we measured stress and anxiety, as subjective stress markers. Significant changes in the level of salivary $\alpha$-amylase were found in response to stress induced xerostomia. However, the correlation of salivary $\alpha$-amylase with corticosterone had only partial statistical significance. In conclusion, We found that salivary $\alpha$-amylase and corticosterone were sensitive to stress induced xerostomia throughout this study. Thus, salivary $\alpha$-amylase and corticosterone may be used to measure stress through xerostomia non-invasively in both clinical settings and nursing research studies where the effects of stress might be scrutinized. Furthermore, the mechanisms of xerostomia that are induced by stress should be explored.

REFERENCES


