唾液の臨床検査　～負荷の強度と唾液中クロモグラニン A 量の関係に関する研究～

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Clinical Testing using Saliva: A Study on the Relationship between the Load Intensity and the Amount of Salivary Chromogranin A

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臨床検査検体としての唾液

Saliva as a Clinical Laboratory Specimen

Summary

The aim of this research was to address the potential usefulness of saliva as a noninvasive, easy-to-collect clinical specimen. Specifically, in this study, salivary Chromogranin A(CgA) was examined as a potential indicator of stress related to daily-life activities. In this preliminary investigation the stress related to a walking exercise and a palm massage was assessed.

As a result of walking exercise (intensity: 3.5-4.0 METs), the maximum increase for subjects with intermediate baseline CgA levels ranged from 65 % to 270%, while the maximum increase for subjects with high baseline CgA levels ranged from 51% to 87%. As a result of palm massage (intensity: 1.3 METs), the maximum increase for subjects with intermediate baseline CgA levels ranged from 10% to 150%. In 5 subjects, palm massage reduced CgA levels. The increase observed with walking exercise was higher than with palm massage.

These results suggest that salivary CgA levels tend to increase in response to behavioral loads in daily life and that the rate of increase is related to the intensity of stress, therefore supporting its potential application as a marker of stress.

Keywords.

saliva, Chromogranin A (CgA), stress

Introduction

In clinical laboratories, most clinical tests are based on biomarkers collected from blood or urine. However, blood sampling is somewhat invasive whereas urine sampling, although non-invasive, can be difficult in specific groups of patients, for example in those with urinary difficulties. In addition, results from urine sampling are affected by renal function and the obtained biomarkers may not accurately reflect in vivo changes in patients with impaired renal function, such as elderly patients. As a potential alternate approach to blood and urine sampling, saliva sampling could be considered as a non-invasive, less burdensome, and potentially more accurate in terms of providing in vivo information. In this study, the potential of saliva as a sample for clinical testing is investigated.

Salivation is an exocrine secretion into the oral cavity resulting in a volume of saliva of more than 1 liter per day in adults. Saliva is composed for more than 99% of water, whereas the remaining 1% includes organic components such as amylase and mucin, and very small amounts of inorganic components such as Sodium (Na), Chlorine (Cl). Salivary secretion is under the dual control of the sympathetic and parasympathetic nervous systems. In general, the sympathetic and parasympathetic nervous systems are antagonistic to each other, but salivary secretion represents an exception. Specifically, the action of the sympathetic nervous system is exerted through the neurotransmitter noradrenaline. Noradrenaline binds to receptors on the salivary secretory cells, causing the outer membrane of the amylase-containing secretory granules to fuse with the cell membrane of the lumen of the gland and, therefore, causing the content to flow into the lumen of the gland. This effect is stronger via beta receptors than via alpha receptors. The action of the parasympathetic nervous system is mediated by the neurotransmitter acetylcholine that binds to muscarinic receptors on secretory cells, resulting in the secretion of water and saliva[1].

There are advantages and disadvantages in using saliva as a specimen for clinical testing. Compared to blood samples, collection of saliva is easier and non-invasive. Collection of saliva can be difficult in elderly subjects and in patients with xerostomia, but to compensate for these issues saliva secretion can be stimulated by placing a saliva collection roll into the oral cavity and by asking the subject to chew it[2]. Another advantage is that saliva is easy to handle as it contains few cellular components such as erythrocytes, leukocytes and platelets and it does not coagulate. On the other hand, saliva is an exocrine fluid and therefore, unlike blood which has mechanisms to maintain homeostasis, it is prone to deterioration, for example when stored at room temperature. Salivary secretion is known to fluctuate during the day, both in terms of secretion volume and in terms of composition, particularly total protein content. For this reason, it has been suggested that the mass of total protein in saliva should be measured simultaneously and used to derive a relative amount of salivary constituents, i.e. a relative value per mg of total protein.

Saliva testing is therefore more suitable for assessing intraindividual changes over time than for comparing measures taken in different subjects.

Stress can be physical or psychological. Physical stress is transmitted directly to the hypothalamus, whereas psychological stress is transmitted to the hypothalamus via the cerebral cortex and the limbic system. The response of the organism to stress can be divided into two types: the endocrine response of the hypothalamic-pituitary-adrenocortical system and the autonomic response of the sympathetic-adrenal medullary system[3].

The present study investigates the variability of a specific stress marker, i.e., chromogranin A (CgA) in saliva in relation to exogenous stress from low intensity daily life activities, in order to preliminarily investigate the potential of saliva as a sample for clinical examination. CgA is an acidic glycoprotein of 439 amino acids that is stored and co-secreted with catecholamines by the adrenal medulla and by the sympathetic nerve endings[4]. Salivary CgA is produced in the submandibular gland and released into the salivary glands upon autonomic nervous system stimulation and it is considered a marker of the activity of the sympathetic-adrenal system[5]. It has been reported that salivary CgA can be used as a marker of short-term mental stress, and also that chronic mental stress may affect the response of the sympathetic-adrenal medullary system[6]. Salivary CgA has also been reported to be a useful indicator of physical stress[7].

Based on the evidence from the above mentioned study, the present study investigates the relationship between the intensity of stress and the amount of salivary CgA in relation to two exemplary low intensity loads from daily life activities: (1) walking and (2) palm massage.

Materials and Methods

In this study, the amount of salivary CgA was computed as a relative value obtained by dividing the CgA amount (pmol) by the mass of total protein (TPmg) and expressed in relative amount of CgA (pmol/TPmg). Reagents were purchased from Fujifilm Wako Pure Chemicals Corporation (Osaka, Japan) unless otherwise specified.

1) Collection of saliva

Saliva was collected after gargling with tap water using Salivettes (cotton, white cap, SARSTEDT Corporation, Tokyo, Japan). The rolled cotton was placed in the oral cavity, the cotton was chewed once with the upper and lower back teeth for 2 min, then the rolled cotton was returned to the tube, centrifuged at 1690 x g for 2 min, and the saliva (supernatant) was collected.

2) Measurement of total protein (TP) concentration in saliva

The concentration of total protein (TP) in saliva was measured using a Biuret reagent (prepared in-house).

For the Biuret reagent, 1.5 g of $CuSO_4 \cdot 5H_2O$ and 6.0 g of $C_4H_4KNaO_6 \cdot 4H_2O$ were dissolved in approximately 500 mL of distilled water, and 30 g of NaOH and 1.0 g of KI were added. After dissolving, distilled water was further added to make 1 L solution. The Biuret reagent was stored in a light-proof bottle at room temperature.

The sample and reagent volumes were adjusted and the concentration of TP in saliva was measured by the procedures 1-6 described in the following. The measurement was confirmed by calibration curves (Figure 1).

1. 2.0 mg/mL albumin standard solution was prepared by dissolving human serum albumin in physiological saline solution.

2. 2.0 mg/mL albumin standard solution was diluted with saline to prepare 1.5 mg/mL, 1.0 mg/mL and 0.50 mg/mL albumin standard solutions.

3. 150 µL of standard solution or sample (saliva) was added to each well of a 96-well microplate.

4. 100 µL of Biuret reagent was added to the wells containing the standard or sample and the microplate was shaken at 100 rpm for 15 min at room temperature to allow the reaction to occur.

5. Absorbance was measured at 540 nm using a microplate reader (SH-1200Lab) (Corona Electric Co Ltd, Sanjo, Niigata, Japan).

6. The concentration of TP in saliva was calculated from the calibration curve (Figure 1).

3) Measurement of salivary CgA concentration

The concentration of CgA in saliva was used using a Human Chromogranin A EIA Kit (Yanaihara Laboratories, Fujinomiya, Shizuoka, Japan).

4) Calculation of salivary CgA content

The amounts of CgA and TP were calculated by multiplying the CgA and TP concentrations by the saliva volume, and the amount of CgA was corrected for TP by dividing the amount of CgA by the amount of TP.

5) Target load content

1. 30-minute walking load

Five female students (age: 21 years) from the Department of Medical Laboratory Science, Faculty of Health Science, Kobe Tokiwa University, who were given informed consent, were included as participants. To estimate baseline CgA levels, saliva samples were collected during a resting period. Participants were then asked to perform a 30-minute walking task (including stair climbing) around the university campus, and saliva was collected after 5 min, 10 min, and 30 min from the onset of exercise, and 5 min, 10 min, 30 min, and 40 min after the offset of exercise (Figure 2). Loading and saliva collection were performed simultaneously on 5 subjects. The intensity of the exercise, expressed in metabolic equivalents (METs) with

reference to the METs table of physical activity of the National Institute of Health and Nutrition[8], ranged from 3.5 to 4.0 METs, specifically an intensity of 3.5 METs was assumed for walking, 3.5 METs for walking down the stairs, and 4.0 METs for walking slowly up the stairs.

2. 30 min palm massage load

Seventeen students (aged 21-22 years, 15 females, 2 males) from the Department of Medical Laboratory Science, Faculty of Health Sciences, Kobe Tokiwa University who were given informed consent were included in the study.

To estimate baseline CgA levels, saliva samples were collected before the massage. Saliva was then collected immediately after the start of loading at the following instants: 0 min, 5 min, 10 min, and 30 min (Figure 3). The massage was performed with the subject sitting in a chair, massaging their own palms with one thumb for 15 min per hand. The intensity of the massage was estimated to be 1.3 METs, with 1.3 METs corresponding to writing (in a seated position) and 1.3 METs corresponding to haircut and nail art by somebody else (in a seated position).

6) Analysis

Measurement data were analyzed with t-test using IBM SPSS Statistics (IBM Corporation, Tokyo, Japan). Significant differences were examined by Kruskal-Wallis test. P value < 0.05 was considered significant.

7) Ethics

This study was conducted with the approval of the Research Ethics Committee of Kobe Tokiwa University. (Approval No.: Kobe Tokiwa University Research Institute Ethics No. 22-1).

Results

Variation in salivary CgA levels due to walking stress

Figure 4 shows the distributions of salivary CgA levels observed before, during, and after exercise (walking) in the five participants. No significant differences were observed between the mean relative values of salivary CgA before, during and after exercise (P value > 0.05).

The subjects whose baseline CgA levels were at an intermediate level (i.e., between 1.8 pmol/TPmg and 22.5 pmol/TPmg) showed a maximum increase in the range 65-270%, whereas those with higher CgA level (above 22.5 pmol/TPmg or more) showed a maximum increase in the range 51-87% (Table 1). On average, subjects with intermediate baseline CgA level showed a higher rate of increase than the subjects with high baseline CgA levels. (Based on the mean and SD of the CgA levels we previously determined from 77 saliva samples, we grouped the low values into less than 1.8 pmol/TPmg, intermediate values into between 1.8 pmol/TPmg and 22.5 pmol/TPmg, and high values into 22.5 pmol/TPmg or higher.)

In addition, three subjects (A, C and D) showed the maximum rate of increase at 10 minutes under load. There was one load going up the stairs (15 steps) by 10 minutes during the load, but there were three loads going up the stairs (22, 3 and 23 steps) between 10 and 30 minutes during the load. The results showed that the CgA levels were not increased due to the load going up the stairs.

Variation in salivary CgA levels due to palm massage

Figure 5 shows the distributions of salivary CgA levels observed before and during exercise (palm massage) in the 17 subjects. No significant differences were found between the mean relative values of salivary CgA before and during loading (P value > 0.05).

All subjects had intermediate baseline CgA values. The maximum increase was in the range 10-150% (Table 2). Some subjects showed a decrease in salivary CgA levels, up to -64%.

Comparison of walking load and palm massage load

The maximum rate of increase in walking induced stress ranged from 51 to 270% in subjects whose preload values were intermediate, whereas the maximum rate of increase in palm massage induced stress ranged from 10 to 150%. On average, the rate of increase was higher for the walking load compared to palm massage load.

For the walking induced stress, 3 out of 5 subjects (i.e., 60%) had the highest rate of increase at 10 minutes during walking, 1 out of 5 subjects (20%) had the highest rate of increase at 30 minutes during walking, and 1 out of 5 subjects (20%) had the highest rate of increase at 30 minutes after walking. For the palm massage induced stress, 4 out of 17 subjects (24%) had the highest rate of change at 0 minute (immediately after the start of massage), 4 out of 17 subjects (24%) had the highest rate of increase after 5 minutes of massage, 1 out of 17 subjects (6%) had the highest rate of increase after 10 minutes of massage, 3 out of 17 subjects (17%) had the highest rate of increase after 30 minutes of massage and in 5 out of 17 subjects (29%) the CgA decreased at all time points. There was a trend for earlier response to palm massage compared to walking. (Table 2, Table 4)

Discussion

This study focused on the potential of saliva as a clinical testing sample that can be easily collected non-invasively. In the present study, we simulated low intensity daily-life loading using walking and palm massage tasks, and we investigated changes in the amount of salivary CgA over time in response to these tasks.

Results showed that the rate of increase in salivary CgA was higher when the baseline (pre-load) value was intermediate compared to high baseline values, and that salivary CgA tended to be less responsive to exercise (walking) when the pre-load value was high. Moreover, results showed that salivary CgA levels increased to a larger extent during walking (estimated load intensity: 3.5-4.0 METs) than during palm massage (estimated load intensity: 1.3 METs). In addition, salivary CgA decreased in some subjects during palm massage, suggesting that those subjects may have felt relaxed by the massage. Overall, the results suggest a relationship between the intensity of the exercise and the observed increase in salivary CgA.

In the walking load, subject C had a particularly high pre-load value of 90.2 pmol/TPmg. A questionnaire (whether feeling tired, stressed, nutritional status/dietary habits, physical condition, mood) was taken prior to saliva collection, and subject C responded no fatigue, no stress, normal nutritional status, good physical condition and good mood. The amount of CgA in saliva collected in the pre-experiment was 5.9 pmol/TPmg for subject A, 7.1 pmol/TPmg for subject B, 13.6 pmol/TPmg for subject C, 2.3 pmol/TPmg for subject D and 8.9 pmol/TPmg for subject E.   The mean of the intermediate group (subjects A, B and D) was 5.1 pmol/TPmg and the mean of the high group (subjects C and E) was 11.2 pmol/TPmg.   Subject C showed higher values than the other subjects in the pre-experiment, suggesting that the subject is in fact under stress although not feeling stressed subjectively. In addition, the overall CgA levels in the pre-experiment were lower than before the walking load, suggesting that the subjects may be under stress from the upcoming load.

In our previous experiments, a group with exercise habits (exercise twice a week for 1-2 hours, n=6) and a group without exercise habits (n=8) were loaded with approximately 700 m of walking, and the group with exercise increased their CgA concentration by 13% and the group without exercise increased their CgA concentration by 42% compared to before loading. The CgA levels of the chronic fatigue group (n=5) and the non-chronic fatigue group (n=5) were also measured with regard to fatigue levels. The results showed no significant difference between the chronic fatigue group (2.7 pmol/mL) and the non-chronic fatigue group (3.2 pmol/mL). The level of fatigue was determined using the Ministry of Health, Labour and Welfare's Self-Diagnostic Checklist for Fatigue Accumulation[9]. In addition, CgA concentrations were measured before and after the presentation of the study with regard to neurological stress (n=12). The mean CgA concentration before the study publication was 8.7 pmol/mL and after the study publication was 17.5 pmol/mL, with an increase in CgA concentration of +8.8 pmol/mL (101%) after the publication compared to before. Although each subject was subjected to the same load in the present study, previous experimental results suggest that CgA levels may vary depending on the individual subject's background, and the stress level before the load may also differ. In addition, in walking loads, the intensity of the load may differ depending on the subject's

body weight.

On the whole, the results of this study suggest that: (1) the response to the load, in terms of measured increase in CgA, tends to be lower when there is a relatively high level of stress before the load, (2) the amount of CgA in saliva increases to a larger extent when the intensity of the load is higher, and (3) the amount of CgA in saliva may reflect the degree of individual relaxation.

The results also showed that the amount of CgA in saliva tends to fluctuate according to the intensity of the load. In the future, it will be important to investigate the relationship between the intensity of the load and the fluctuations in different salivary components in order to expand the range of applications of saliva as a possible clinical test specimen.

The present study has the following limitations; the study sample was small and most of the subjects were young female in their early 20s from a university in the Kansai region. In addition, the types of exercise (load) were limited to two: walking and massage. Therefore, studies with a wider age range of males and females and patients with underlying diseases will be needed to investigate more deeply intra- and inter-individual differences in CgA levels as a function of exercise and individual characteristics.

Acknowledgement

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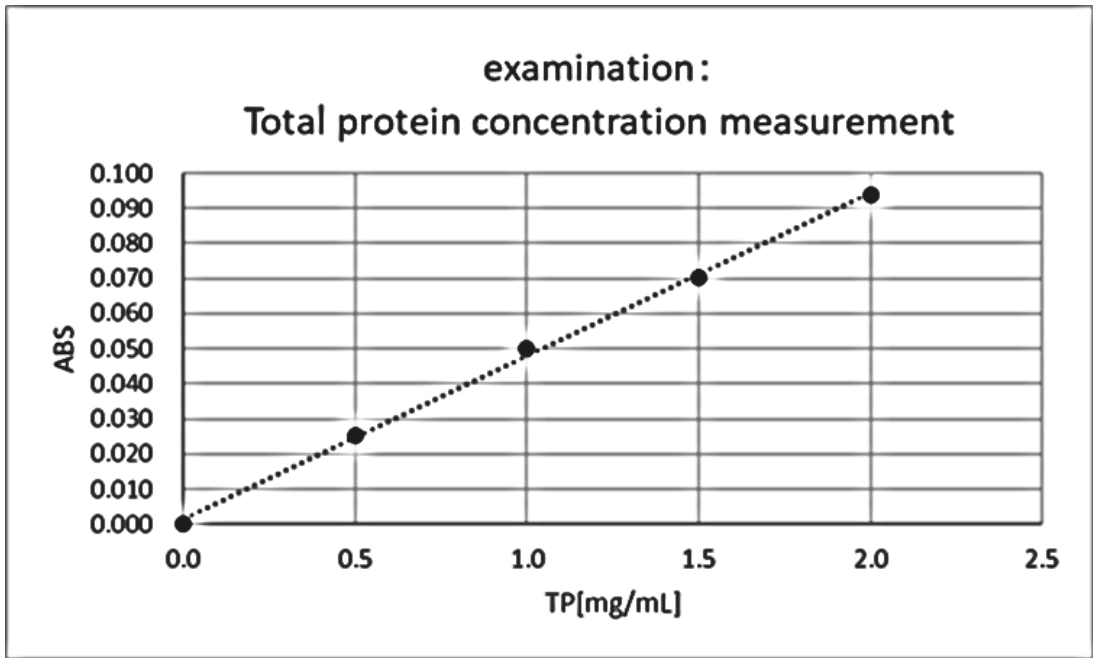
Figure 1 Calibration curve (TP-ABS)

Calibration curve constructed with albumin standard solution concentration (TP concentration) on the horizontal axis and absorbance (ABS) on the vertical axis.
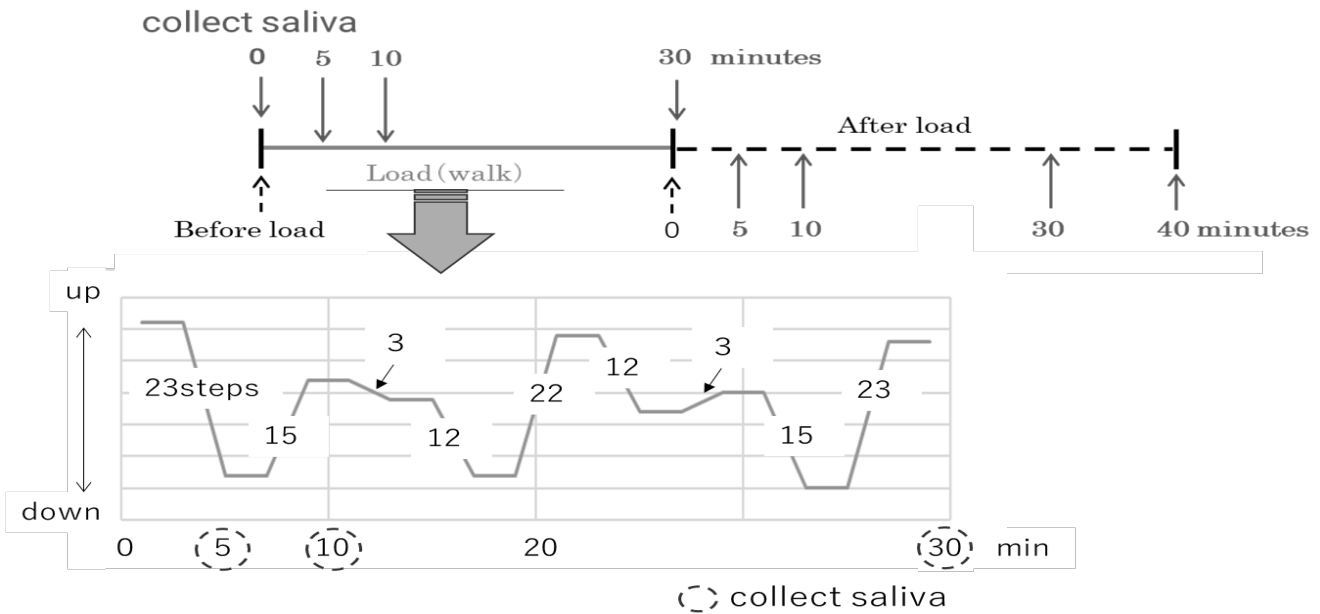

Figure 2 Timing of saliva collection (load: walk)

Saliva was collected at rest (0 min loading), and after 5 min, 10 min, and 30 min during loading (which coincides with 0 min after loading), and then 5 min, 10 min, 30 min, and 40 min after loading.
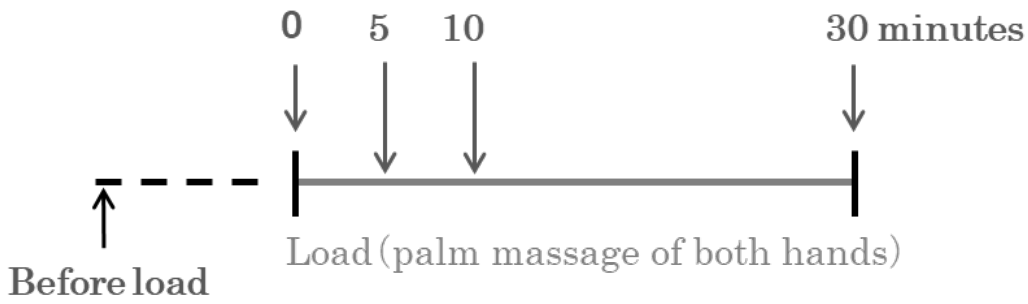
Figure 3 Timing of saliva collection (load: palm massage)

Saliva was collected before loading and 0 min, 5 min, 10 min, and 30 min during loading.
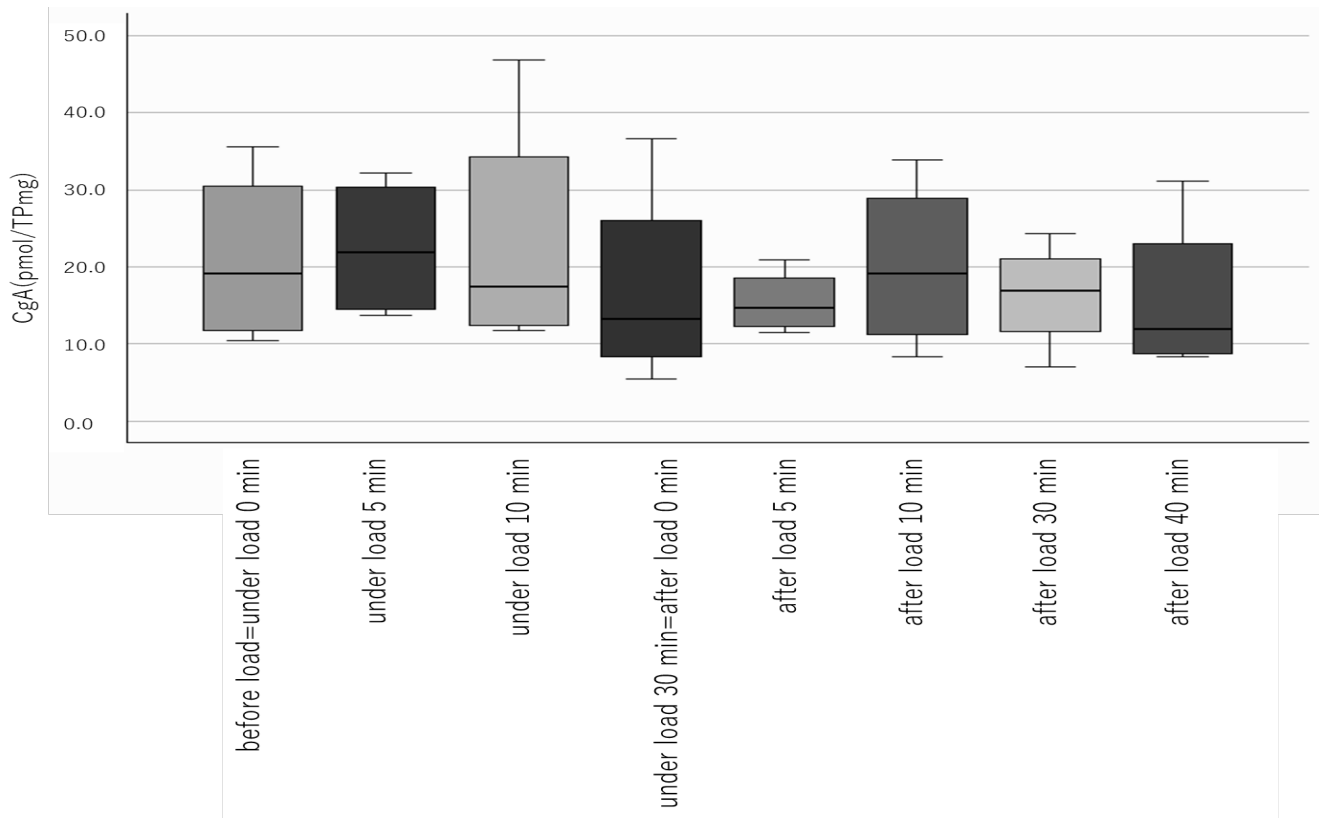
Figure 4 Variation of CgA with load (load: walk)

This figure shows the distributions of salivary CgA levels observed before, during, and after exercise (walking) in the five participants. No significant differences were observed between the mean relative values of salivary CgA before, during and after exercise.   P value >0.05
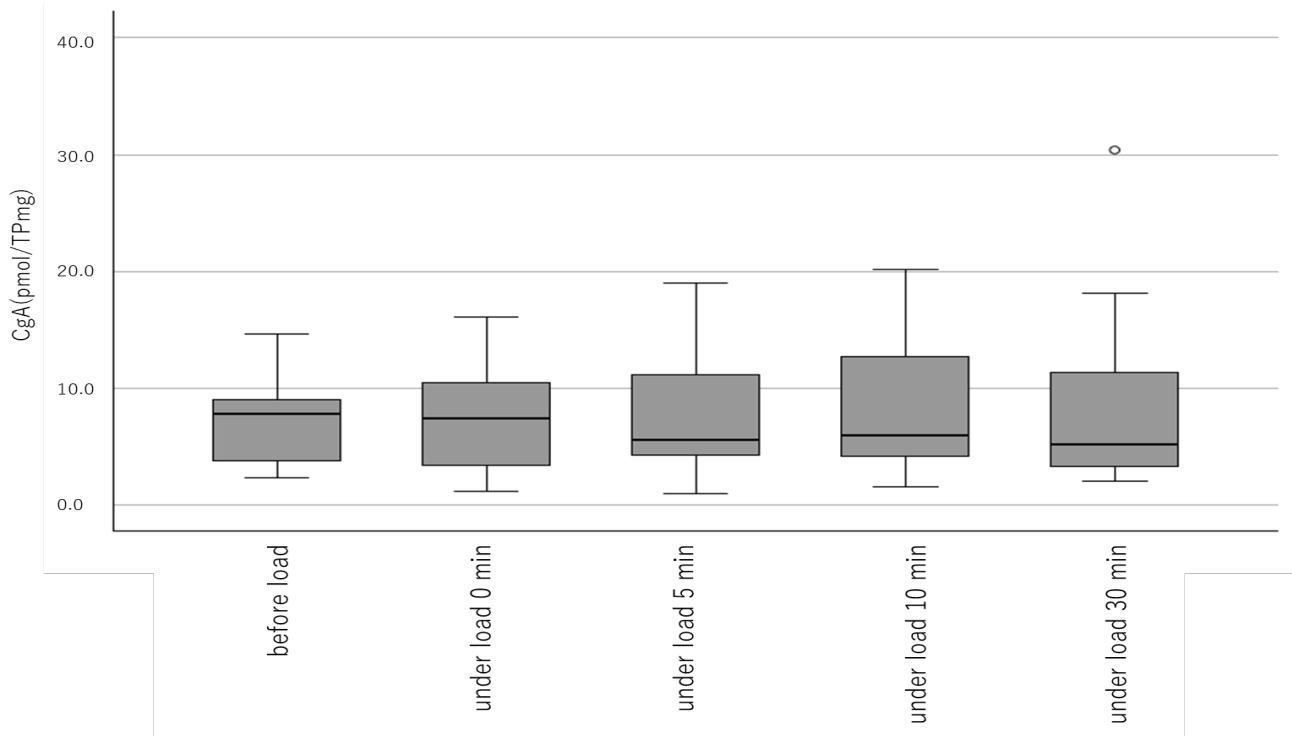
Figure 5 Variation of CgA volume with load (load: palm massage)

This figure shows the distributions of salivary CgA levels observed before and during exercise (palm massage) in the 17 subjects. No significant differences were found between the mean relative values of salivary CgA before and during loading.   P value > 0.05

Table 1 Variation of CgA content with load (load: walk)

Percent change in CgA relative amount compared to the baseline value in each of the five participants.

	before load =Under load 0 min CgA[pmol/TPmg]		under load 5 min CgA[pmol/TPmg]	%	under load 10 min CgA[pmol/TPmg]	%	under load 30 min = after load 0 min CgA[pmol/TPmg]	%	after load 5 min CgA[pmol/TPmg]	%	after load 10 min CgA[pmol/TPmg]	%	after load 30 min CgA[pmol/TPmg]	%	after load 40 min CgA[pmol/TPmg]	%
A	8.3	intermediate	10.5	27	13.7	65	11.7	41	5.5	-34	11.5	38	8.3	-1	7.1	-15
B	9.1	intermediate	13.0	42	15.2	66	21.8	138	15.3	67	16.3	78	33.9	270	17.8	94
C	90.1	high	161.3	79	168.9	87	148.5	65	106.0	18	63.4	-30	74.9	-17	–	–
D	14.9	intermediate	25.2	69	32.2	116	13.0	-13	11.1	-25	13.1	-12	14.2	-5	16.1	8
E	31.1	high	35.6	15	28.5	-8	46.8	51	36.6	18	20.9	-33	24.0	-23	24.3	-22
average	30.7		49.1	60	51.7	68	48.4	57	34.9	14	25.0	-18	31.1	1	16.3	-47

Table 2　Variation of CgA volume with load (load: palm massage)

Percent change in CgA relative amount compared to the baseline value in each of the 17 participants.

	before load		under load 0 min		under load 5 min		under load 10 min		under load 30 min	
	CgA[pmol/TPmg]		CgA[pmol/TPmg]	%	CgA[pmol/TPmg]	%	CgA[pmol/TPmg]	%	CgA[pmol/TPmg]	%
A	8.0	intermediate	12.6	58	13.5	69	20.0	150	18.1	126
B	10.1	intermediate	10.1	0	9.7	-4	7.5	-26	14.5	44
C	13.7	intermediate	14.6	7	11.2	-18	20.2	47	30.4	122
D	2.3	intermediate	1.9	-17	2.8	22	1.9	-17	3.3	43
E	8.9	intermediate	16.1	81	14.2	60	15.1	70	11.4	28
F	3.7	intermediate	6.7	81	4.3	16	4.2	14	4.0	8
G	14.7	intermediate	10.3	-30	9.2	-37	12.7	-14	7.6	-48
H	3.8	intermediate	3.4	-11	2.4	-37	1.9	-50	2.1	-45
I	4.0	intermediate	2.5	-38	5.6	40	3.2	-20	2.2	-45
J	13.5	intermediate	14.9	10	19.0	41	17.5	30	15.1	12
K	8.2	intermediate	9.0	10	5.3	-35	8.2	0	6.8	-17
L	7.3	intermediate	5.8	-21	7.2	-1	6.0	-18	6.8	-7
M	7.8	intermediate	10.5	35	11.4	46	10.8	38	5.2	-33
N	5.7	intermediate	7.4	30	1.9	-67	4.2	-26	3.5	-39
O	2.6	intermediate	2.1	-19	5.1	96	4.5	73	2.9	12
P	2.8	intermediate	1.2	-57	1.0	-64	1.6	-43	2.3	-18
Q	9.0	intermediate	7.4	-18	5.5	-39	5.5	-39	3.6	-60
average	7.8		8.8	15	7.8	0.1	9.4	21	9.1	18