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<td>Author(s)</td>
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Influence of Chewing Force on Salivary Stress Markers as Indicator of Mental Stress

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The aim of the present study was to investigate the influence of chewing force on salivary stress markers (alpha-amylase activity, salivary cortisol level, and secretory immunoglobulin A secretion rate) as indicators of mental stress. Participants comprised 20 healthy men. The first set of saliva specimens (S1) was collected at immediately after a 20-minute rest to evaluate stress markers. As stress loading, the participants were required to perform arithmetic calculations for 20 min, after which the second set of saliva specimens (S2) was collected. Each participant was then required to chew a piece of tasteless gum for 10 min, after which the third set of saliva specimens (S3) was collected. After a 20-minute rest, the fourth set of saliva specimens (S4) was collected. Weak, habitual, and strong chewing forces were assigned. Change rates of stress markers between S2 and S3, and S2 and S4 were calculated. A significant difference was observed in the change rate of cortisol levels between S2 and S3. Cortisol level decreased more under strong chewing than under weak chewing. No significant differences were observed in the change rate of amylase activity or s-IgA secretion rate among the 3 chewing forces. The results suggest that differences in chewing force influence the salivary cortisol level of the 3 stress markers, and that a strong chewing force induces a greater reduction in mental stress than a weak one.
Introduction

Many reports have been published on mental stress reduction by chewing as reflected in changes in sympathetic, endocrine, and immune system markers in saliva. Nakajo et al. reported that alpha-amylose activity, a sympathetic nervous system stress marker, decreased with chewing in a mentally stressful environment (1). Tahara et al. (2) and Scholey et al. (3) reported that salivary cortisol levels, an endocrine system stress marker, decreased with chewing after mental stress loading. Furthermore, Ishiyama et al. reported that s-IgA levels, an immune system stress marker, decreased with chewing (4).

In terms of masticatory movement parameters, Tasaka et al. suggested that a fast chewing rate induced a greater reduction in mental stress than a slow one (5). No studies, however, have investigated the influence of other masticatory movement parameters such as chewing force, chewing number, and chewing time on reduction of mental stress. Peter A et al. reported a correlation between the mean amplitude electromyogram (EMG) of the masticatory muscle and chewing force (6). Ruf S et al. reported that the mean amplitude EMG of the masticatory muscle during chewing increased under mental stress (7). Niwa M et al. reported that chewing increased activity in the prefrontal cortex, which is involved in stress control (8).
We hypothesized that chewing force as a masticatory movement parameter induced a reduction in mental stress. The aim of the present study was to investigate the influence of chewing force on salivary stress markers (sympathetic system alpha-amylase activity, endocrine system salivary cortisol level, and immune system s-IgA secretion rate) as indicators of mental stress.

Materials and methods

Participants

Twenty healthy men of between 23 and 30 years of age (average, 25.5 years) were recruited from students and staff at Tokyo Dental College. All participants had complete natural dentition, excluding the third molars, and were without subjective or objective abnormalities of the stomatognathic system. None had any medical history of mental illness. All were non-smokers. Written informed consent was obtained from all participants. The study was approved by the Ethics Committee of Tokyo Dental College (#218).

Experiment schedule
In consideration of the influence of circadian rhythm on salivary stress marker levels, the experiments were performed between 2:00 PM and 7:00 PM. The participants were required to refrain from eating, drinking (alcohol, caffeine), taking drug and exercising within 2 hr prior to commencement of the experiments (9, 10) and were allowed 20 min in the experimental room to rest and familiarize themselves with the environment before testing. Immediately after this, the first set of saliva specimens (S1) was collected. As stress loading, participants were required to perform arithmetic calculations (addition, subtraction, multiplication, division) for 20 min, after which the second set of saliva specimens (S2) was collected. Each participant was then required to chew a piece of tasteless gum base weighing 1.0 g (Lotte, Saitama, Japan) for 10 min. The hardness of the gum base was $6.4 \times 10^3$ Pa $\cdot$ s (soft type). After that, the third set of saliva specimens (S3) was collected. After a 20-min rest, the fourth set of saliva specimens (S4) was collected, completing the experiment (Fig. 1). The schedule of mental stress loading and rests was determined based on previous reports (2, 5).

Weak, habitual, and strong chewing forces were applied. Participants were told to chew softly for weak chewing, and strongly for strong chewing. Conscious chewing does not cause discomfort or fatigue of the masticatory muscle. Therefore, a non-chewing condition was not applied to avoid excessive stress.
The habitual chewing rate of each participant was determined with an electronic metronome.

The participants were instructed to maintain the same posture throughout the experiment. Each participant was assigned all 3 chewing forces, each on a different days, also left the interval more than a day. The 3 chewing forces were assigned in random order.
Measurements

In this study, salivary cortisol, alpha-amylase and s-IgA levels were selected as markers of increase in stress. The cortisol and s-IgA saliva specimens were collected using a saliva sampling device (Salivette, Sarsted, Rommelsdorf, Germany), keeping cotton rolls in the oral cavity for 2 min. In the laboratory, the samples were centrifuged at 3000 rpm for 20 min in a refrigerated centrifuge. The supernatant of the collected whole saliva was frozen at -20°C. Salivary cortisol levels (μg/dl) were determined with a radioimmunoassay kit (GammaCoat, Diasorin, Stillwater, OK, USA) in accordance with the manufacturer’s instructions. The s-IgA levels (μg/dl) were determined with an enzyme immunoassay kit (Poseidon2, Aloka, Tokyo, Japan). Alpha-amylase saliva specimens were collected with an amylase monitor chip (Salivary amylase monitor chip, Nipro, Osaka, Japan). The monitor chip was immersed in whole saliva under the tongue of the participant for 30 sec.

Alpha-amylase activity (kU/l) was analyzed with a salivary amylase monitor (Salivary Amylase Monitor, Nipro, Osaka, Japan). The s-IgA level (μg/dl) was corrected to the s-IgA secretion rate (μg/min) by saliva flow rate for 2 min (dl). This was because, while salivary cortisol level and alpha-amylase activity are not influenced by saliva flow rate, s-IgA level is (11–16).
This was because, while salivary cortisol level and alpha-amylase activity are not influenced by saliva flow rate (11-14). S-IgA level is influenced (15, 16).

An EMG recording system (Muscle Tester ME3000P, Mega Electronics, Kuopio, Finland), with a built-in 12-bit A/D converter was used to monitor chewing rates and determine myoelectrical activity in the masseter muscle. For EMG of muscle activity, bipolar surface electrodes (Blue Sensor P-00-S, Medicotest, Olstykke, Denmark) were positioned parallel to the main direction of the muscle fibers on the maximal bulk of the bilateral masseter muscle, which was determined by palpation while the participants clenched intermittently. Interelectrode distance was 25 mm centre-to-centre. Reference (grounding) electrodes with integrated preamplifiers were attached behind the ear. Prior to attachment of the electrodes and bioelectrical measurement, the skin was thoroughly cleansed with a specific skin cleansing gel (Skin Pure, Nihon Kohden, Tokyo, Japan) and ethanol-soaked gauze. Skin impedance between the electrodes was lower than 8 kΩ. Sampling frequency was 1000 Hz at a sampling period of 0.1 sec. The daily precision of the EMG was corrected by the mean amplitude EMG of the masseter muscle under non-chewing conditions.

Statistical analysis
Alpha-amylase activity at S1 was regarded as signifying the baseline relaxed state. Participants showing a lower alpha-amylase activity at S2 than at S1 were regarded as being uninfluenced by the mental stress loading applied in this study and excluded from the statistical analysis. Changes in salivary stress markers (salivary cortisol level, alpha-amylase activity and s-IgA secretion rate) between S2 and S3, and S2 and S4 were determined and compared. The mean amplitude and integrated EMG of the masseter muscle over 10 min was calculated. Changes in salivary stress markers and the mean amplitude and integrated EMG of the masseter muscle among the 3 chewing forces were compared using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5% using statistical software (SPSS 11.0J; SPSS, Chicago, IL, USA).

Results

Figure 2 shows changes in salivary cortisol level with time. Change in the salivary cortisol level was expressed as a change rate, as individual differences exist in cortisol levels. The change in the salivary cortisol level between S2 and S3 was significantly smaller with strong chewing than with weak chewing. Only the change rate in salivary cortisol level with weak chewing increased, suggesting an increase in mental stress. The change in salivary cortisol level between
S2 and S4 decreased under all chewing conditions, but no significant difference was observed among the 3 chewing forces (Fig. 3).

Figure 4 shows change in alpha-amylase activity with time. The mean change rates of alpha-amylase activity between S2 and S3, and S2 and S4 decreased with strong chewing and increased with weak chewing, but no significant difference was observed among the 3 chewing forces (Figs. 5).

Figure 6 shows change in s-IgA secretion rate with time. The change in the s-IgA secretion rate between S2 and S3 decreased under all chewing conditions. No significant difference was observed among the 3 chewing forces. Change in the s-IgA secretion rate between S2 and S4 decreased with weak and habitual chewing and increased with strong chewing. No significant differences were observed among the 3 chewing forces (Fig. 7).

The mean amplitude EMG of the masseter muscle showed significant differences among the 3 chewing rates (Fig. 8). Comparison of the mean amplitude EMG revealed that strong chewing comprised 169% of habitual chewing, and weak chewing 25%. The integrated EMG of the masseter muscle revealed significant differences among the 3 chewing rates (Fig. 9).

Discussion
Individual differences were observed in the level of chewing force required to fatigue each participant in a pilot experiment. Therefore, chewing conditions were determined by the participants themselves to avoid physical stress. The participants were instructed to adjust the chewing force consciously. Mean amplitude EMG is considered to be associated with chewing force. Significant differences in the mean amplitude EMG of the masseter muscle were observed under all chewing conditions, thus confirming differences in chewing force and the validity of setting different chewing forces as an experimental condition. Although chewing force can be adjusted by changing the hardness of the gum base, this method was not applied in the present study, as occlusal force differs among individuals and recognition of softness and change in gum base hardness in itself may cause physical stress.

In the pilot study, change in cortisol level, alpha-amylase activity, and s-IgA secretion rate in saliva after mental stress loading was compared between with and without chewing. The results showed that stress markers in saliva were lower under chewing than non-chewing conditions. This was consistent with the results of earlier reports (1-4). In the present study, taking stress into consideration, the change rate was compared without applying a non-chewing condition (there was a 10-min rest without chewing after stress loading).
The change rate in salivary cortisol level between S2 and S3 showed a significantly greater decrease with strong chewing compared with weak chewing. This suggests that strong chewing reduces mental stress more than weak chewing.

Stress includes both mental and physical stress. In the present study, arithmetic calculation (addition, subtraction, multiplication, and division) was used to apply mental stress. It was confirmed that alpha-amylase activity was higher at S2 (after mental stress loading) than at S1 (before mental stress loading). It has been reported that alpha-amylase, a sympathetic system stress marker, reacts rapidly, and is therefore considered to be an effective mental stress marker (17). Four of 20 participants in this study showed lower alpha-amylase activity at S2 than at S1, and these were excluded from the statistical analysis.

Salivary cortisol level was endocrine system stress marker. In this study all participants were male. Because, female participants need to control estradiol cycle (18). If it were not controlled, daily variance of cortisol level was large.

Salivary cortisol level did not increase at S2 compared with at S1 with weak chewing. We believe that this was because salivary cortisol level increases at S1 before stress loading due to stress anticipation (19, 20), and it takes 5-20 minutes for stress-induced blood cortisol to appear
in saliva (12, 21). S-IgA secretion rate and amylase activity are increase immediately after stress loading.

There were reported about circadian rhythm. In cortisol level, circadian variability is low after 3:00 PM (22). In amylase activity, circadian variability is low between 4:00 and 5:00 PM (23). In s-IgA secretion rate, circadian variability is low afternoon (24). Considering all of stress marker’s condition, experimental schedule is severe. In this study, the experiment is performed between 2:00PM and 7:00PM.

And then, there were reported about stress marker reaction time. In cortisol levels, it takes 5-20 minutes for increase after stress loading (12, 21). In amylase activity, it takes 1 minute for increase after stress loading (25). In s-IgA secretion rate, immediately increase after stress loading (26). Considering all of stress marker’s character, saliva was collected immediately after task and each task was set enough long time in this study.

The cortisol-involving hypothalamic-pituitary-adrenal axis (HPA), the amylase-involving sympatho-adrenal system (SAS), and the s-IgA-involving immune system react to mental stress. However, it was reported that the s-IgA reaction under acute mental stress reflects SAS stress reaction (27, 28). The s-IgA secretion rate was reported to increase by acute stress and decrease by chronic stress (29, 30). In this study, it was anticipated that s-IgA secretion rate would
increase with acute stress and decrease with chewing. Previous reports have shown that mental stress is reduced by chewing, as evidenced by a decrease in alpha-amylase activity, a sympathetic nerve marker (1). A number of studies have reported that chewing activates the sympathetic nerves (4, 31, 32). Meanwhile, other reports have demonstrated that chewing activates the parasympathetic nerves (33). In this study, the results showed that using sympathetic nerve activation as a marker of stress reduction was not consistent. Together with the results of the earlier studies mentioned above, this suggests that the reaction changes under different chewing conditions. Although we set the hardness of the sample food, chewing time, and chewing rate, the change rate of alpha-amylase activity varied widely. It is considered difficult to evaluate mental stress reduction based on SAS-related alpha-amylase and s-IgA activity. On the other hand, change in HPA cortisol level showed no relationship with sympathetic nerve activity, and, therefore, no correlation with change in alpha-amylase or s-IgA level (34, 35). This suggests that chewing force specifically affects not the SAS, but the HPA stress reaction.

The integrated EMG of the bilateral masseter muscles (workload) showed a significant difference between weak, habitual, and strong chewing conditions, suggesting that the workload changed under different chewing forces. The relationship between the integrated EMG and salivary cortisol level change rate was analyzed. A moderate negative correlation was observed
in the change rate of the salivary cortisol level and the integrated EMG between S2 and S3 (Fig. 13). The results showed that an increase in chewing force increased the workload and released mental stress. Tasaka et al. reported that there was a correlation between the number of chewing cycles and mental stress reduction ($r = 0.49$). This suggests that conscious strong chewing that does not cause fatigue, and increasing the number of chewing cycles are effective for stress reduction.

Conclusion

The results of the present study showed that chewing force affected an HPA stress marker, salivary cortisol level, and that a strong chewing force was more effective than a weak one in reducing mental stress.

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events are associated with low secretion rates of immunoglobulin A in saliva in the middle aged


Figure legend
Fig 1

Experimental schedule. S was saliva collection timing.

Fig 2

Salivary cortisol level under each condition. These were compared between saliva collection timing in each condition using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig 3

Changes in salivary cortisol level between after stress loading (S2) and after chewing (S3), after stress loading (S2) and after rest (S4). These were compared using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig 4

Alpha-amylase activity under each condition. These were compared between saliva collection timing in each condition using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig 5
Changes in alpha-amylase activity between after stress loading (S2) and after chewing (S3), after stress loading (S2) and after rest (S4). These were compared using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig6

The s-IgA secretion rate under each condition. These were compared between saliva collection timing in each condition using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig7

Changes in s-IgA secretion rate between after stress loading (S2) and after chewing (S3), after stress loading (S2) and after rest (S4). These were compared using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig8

Mean amplitude EMG of masseter muscle for 10 minutes chewing. These were compared using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig9
Integrated EMG of masseter muscle for 10 minutes chewing. These were compared using a one-way repeated measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5%.

Fig10

Correlation between change in salivary cortisol levels between S2 and S3 and integrated EMG of masseter muscle for 10 minutes. This was compared using Spearman’s correlation.
Experimental schedule

Fig1

- Rest
- Stress-loading
- Chewing
- Rest

Time

20 2 20 2 10 2 20 2 min

: Chewing
  a. Weak chewing
  b. Habitual chewing
  c. Strong chewing

S Saliva collection
Salivary cortisol level under each condition

Weak chewing

Habitual chewing

Strong chewing

Fig2

n=16

I: ± SD

NS
Fig3

Change in salivary cortisol level between after stress loading (S2) and after chewing (S3)

Change in salivary cortisol level between after stress loading (S2) and after rest (S4)

n = 16

\[ \text{I : } \pm \text{ SD} \]

* : p<0.05

%
Alpha-amylase activity under each condition

<table>
<thead>
<tr>
<th>Condition</th>
<th>S1</th>
<th>S2</th>
<th>S3</th>
<th>S4</th>
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<tbody>
<tr>
<td>Weak chewing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Habitual chewing</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Strong chewing</td>
<td></td>
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</tbody>
</table>

n=16

\[ \text{±SD} \]

\[ \ast : p<0.05 \]
Fig 5  
Change in alpha-amylase activity between after stress loading (S2) and after chewing (S3)  

\[ n = 16 \]
\[ \bar{X} \pm SD \]
\[ NS \]

Change in alpha-amylase activity between after stress loading (S2) and after rest (S4)

\[ n = 16 \]
\[ \bar{X} \pm SD \]
\[ NS \]
Fig6

s-IgA secretion rate under each condition

μg/min

800
700
600
500
400
300
200
100
0

S-IgA secretion rate

Weak chewing

Habitual chewing

Strong chewing

S1  S2  S3  S4

S1  S2  S3  S4

S1  S2  S3  S4

n=16

\[ \overline{x} \pm SD \]

*: p<0.05
Fig7  Change in s-IgA secretion rate between after stress loading (S2) and after chewing (S3)

n = 16
\[ I \pm SD \]
NS

Change in s-IgA secretion rate between after stress loading (S2) and after rest (S4)

n = 16
\[ I \pm SD \]
NS
Mean amplitude EMG of masseter muscle over 10 min chewing

$\bar{x} \pm SD$

* : $p < 0.05$

$n = 16$
Fig 9

Integrated EMG of masseter muscle over 10 min chewing

Mean integrated EMG of masseter

Weak chewing  Habitual chewing  Strong chewing

n = 16

I : ± SD

* : p<0.05
Correlation between change in salivary cortisol levels between S2 and S3 and integrated EMG of masseter muscle over 10 min

Pearson’s correlation coefficient
\[ r = -0.420 \]
\[ P < 0.01 \]
\[ n = 16 \]