Clinical significance
This study was conducted to clarify the effect of chewing rate on salivary stress hormone levels. The results suggest that the effect on stress release with fast chewing is greater than that with slow chewing.

Abstract
Purpose: The purpose of this study was to clarify the effect of different chewing rates on salivary cortisol levels as a stress indicator.
Methods: The subject group consisted of 16 healthy males. They were required to rest for 30 min, and then given arithmetic calculations to perform for 30 min as stress loading. Immediately after, the first set of saliva specimens (S1) was collected over a period of 1 min to measure cortisol levels. Next, they were asked to chew a tasteless gum base for 10 min, and the second set of saliva specimens (S2) was collected in the same manner. They were then required to rest for 10 min, after which the third set of saliva specimens (S3) was collected. Chewing rates were set to slow, habitual, and fast in time with a metronome. Salivary cortisol levels were analyzed by radioimmunoassay. Changes in salivary cortisol levels comparing S1 with S2, and S1 with S3 were determined.
Results: Changes in salivary cortisol levels between S1 and S2 showed a reduction of 4.7%, 14.6%, and 16.2% with slow, habitual, and fast chewing, respectively. A significant difference was observed between slow and fast chewing. Changes in salivary cortisol levels between S1 and S3 showed a reduction of 14.4%, 22.2%, and 25.8% with slow, habitual, and fast chewing, respectively. A significant difference was observed between slow and fast chewing.
Conclusion: This study showed that differences in chewing rate affected salivary cortisol levels as a stress indicator, and suggested that the effect on stress release with fast chewing is greater than that with slow chewing.

Key words: chewing, cortisol, saliva, stress

Introduction
Masticatory muscle activity and amount and frequency of bruxism have been shown to increase with psychological stress in humans. Bruxism and chewing have been reported to affect the hypothalamic-pituitary-adrenocortical (HPA) axis. Expression of corticotrophin-releasing factor (CRF) increased with restraint stress, and expression of CRF was suppressed by biting in rats, suggesting that bruxism relieves stress by increasing masticatory muscle activity. Tahara et al. have shown HPA axis suppression by chewing and clenching after stress loading using the salivary cortisol levels, which can be simply and non-invasively measured, as an indicator of stress.

Masticatory movements are rhythmical movements generated by a central pattern generator, and are modified by sensory input from peripheral sensors. Oka and Kurachi found that the masticatory movement cycle duration decreases and the degree of jaw opening increases after stress loading. Nakaminami found that masticatory movement rhythm changes after stress loading. These studies have suggested the possibility that stress has an influence on masticatory movement parameters. Based on these observations, we hypothesized that chewing rate as a masticatory movement parameter might influence stress response in humans. The purpose of this study was to clarify the effect of chewing rate on salivary cortisol levels as a stress indicator of the HPA axis.

Materials and Methods
Subjects
Sixteen healthy males between 20 and 33 years of age (average 24.7 years) were selected from students and staff of Tokyo Dental College. All sub-
Projects had complete natural dentition excluding third molars and were without subjective or objective abnormalities of the stomatognathic system. None had any previous medical history of mental illness. The subjects were fully informed about the experimental procedures and informed consent was obtained from all of them. The study was approved by the Ethics Committee of Tokyo Dental College (099).

Experimental schedule (protocol)
In consideration of the circadian rhythm of cortisol levels, the experiments were performed between 2:00 PM and 7:00 PM. Subjects were required to refrain from eating, drinking, and exercising within two hours before the experiments. They were allowed 30 min in the experimental room to familiarize themselves with the environment prior to the experiments. As stress loading, the subjects were required to perform arithmetic calculations (addition, subtraction, multiplication, division) for 30 min. Subsequently, the first set of saliva specimens (S1) was collected. Each subject was then required to chew a piece of tasteless gum base weighing 1.0 g (Lotte, Saitama, Japan) for 10 min, after which, the second set of saliva specimens (S2) was collected. After a 10 min rest, the third set of saliva specimens (S3) was collected. Following another 10 min rest, the fourth set of saliva specimens (SB) was collected, completing the experiment (Fig. 1).

Chewing rates were set to slow, habitual, and fast chewing. The subjects were required to freely chew gum before the experiment to determine habitual chewing rate. We first performed a pilot study to determine a range of chewing rates that would not induce discomfort or masticatory muscle fatigue. Slow chewing was set to 15% slower than habitual chewing, and fast chewing was set to 15% faster than habitual chewing in each individual. Each chewing rate was guided by an electronic metronome. The subjects were instructed to rest by maintaining the same posture during the experiment. Each subject was assigned all 3 chewing rates, a different rate on each day over 3 separate days. The 3 chewing rates were assigned in random order.

Measurements
Saliva specimens were collected using a saliva sampling device (Salivette: Sarsted, Rommelsdorf, Germany), keeping cotton rolls in the oral cavity for 1 min. The supernatant of the collected whole saliva was frozen at ~20°C. Salivary cortisol levels (μg/dl) were analyzed by radioimmunoassay kit (GammaCoat®Cortisol: Diasorin, Stillwater, OK, USA) in accordance with the manufacturer’s instructions.

An electromyographic (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 analyze myoelectrical activity in the masseter muscle. For EMG registration 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 subjects 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 0.1 s.

Statistical analysis
Salivary cortisol levels at SB were regarded as signifying the baseline relaxed state. Subjects showing a lower salivary cortisol level at S1 than at SB were regarded as being uninfluenced by stress loading in this study and were excluded from the statistical analysis. Changes in salivary cortisol levels comparing S1 with S2, and S1 with S3 were determined. The integrated EMG of masseter muscle for 10 min was calculated. Differences in changes in salivary cortisol levels and integrated EMG of masseter muscle among the 3 chewing rates were compared using a one-way repeated-
measures analysis of variance (ANOVA) and the post-hoc Bonferroni test at a significance level of 5% with statistical software (SPSS 11.0J; SPSS, Chicago, IL, USA).

Results
Three of the 16 subjects showed lower salivary cortisol levels at S1 than at SB, and these were excluded from the statistical analysis. Salivary cortisol levels at SB, S1, S2, and S3 showed 0.24 µg/dl, 0.31 µg/dl, 0.30 µg/dl, and 0.27 µg/dl with slow chewing, 0.27 µg/dl, 0.38 µg/dl, 0.31 µg/dl, and 0.29 µg/dl with habitual chewing, and 0.30 µg/dl, 0.45 µg/dl, 0.37 µg/dl, and 0.33 µg/dl with fast chewing. Salivary cortisol levels tended to decrease after stress loading under each rate (Fig. 2). Changes in salivary cortisol levels between S1 and S2 showed a reduction of 4.7%, 14.6%, and 16.2% with slow, habitual, and fast chewing, respectively (Fig. 3). There was a significant difference between slow and fast chewing, with the reduction in salivary cortisol levels with fast chewing being greater than that with slow chewing ($p = 0.024$, $F = 4.121$). Changes in salivary cortisol levels between S1 and S3 showed a reduction of 14.4%, 22.2%, and 25.8% with slow, habitual, and fast chewing, respectively (Fig. 4). There was a significant difference between slow and fast chewing, with the reduction in salivary cortisol levels with fast chewing being greater than that with slow chewing ($p = 0.005$, $F = 6.196$). The integrated EMG of masseter muscle showed no significant differences among the 3 chewing rates (Fig. 5).

Discussion
The present study has shown that differences in chewing rate affect salivary cortisol levels. After stress loading, salivary cortisol levels showed a gradual decrease under all 3 chewing rates. This is consistent with the decrease in salivary cortisol levels after chewing reported by Tahara et al.\textsuperscript{5}
There was a significant difference in changes in salivary cortisol levels among the 3 chewing rates, with the reduction in salivary cortisol levels after fast chewing being greater than that after slow chewing. The reduction in salivary cortisol levels after fast chewing tended to be greater than that after habitual chewing, although the difference was not significant. These results suggest that fast chewing, from among the 3 chewing rates in this study, is the most effective for stress release. Furthermore, we analyzed changes in salivary cortisol levels between S2 and S3 among the 3 chewing rates using a one-way repeated-measures ANOVA. No significant difference was observed in changes in salivary cortisol levels between S2 and S3. This suggests that differences in stress release among chewing rates occurred mainly during the 10-min chewing period. In one study, subjects were reported to experience difficulty in chewing slowly in time with a metronome, but find it easy to chew fast with the metronome. Therefore, slow chewing may have been the more difficult task in this study and was not effective for relieving stress.

There have been a number of varying reports on physiological responses by gum chewing. Ishiyama et al. found that sympathetic hyperactivity increases during gum chewing and parasympathetic hyperactivity increases after gum chewing. Onozuka et al. and Hasegawa et al. found that cerebral blood flow increases during gum chewing. Ohtsuka et al. found that alpha waves increase after gum chewing. These reports suggest that the act of chewing increases sympathetic hyperactivity, and parasympathetic hyperactivity increases after chewing.

In this experiment, salivary cortisol levels were used as an indicator of stress level. It is possible to measure cortisol levels using blood and urine. However, blood collection, being invasive, induces stress. Urine collection is limited by time and place. In contrast, saliva collection offers an easy, non-invasive, and effective alternative. Moreover, salivary cortisol levels are not affected by salivary flow rate.

Salivary cortisol levels have been suggested to increase prior to stress-loading due to anticipation of stress. In consideration of this matter, the salivary cortisol levels at SB were used as the baseline relaxed state in this study. At SB, the subjects had had over 20 min rest after the task had been completed (stress-loading and chewing). The salivary cortisol levels at S1 were different among the 3 chewing rates. In this study, these rates were assigned at random. In addition, there were no significant differences in changes in salivary cortisol levels between S1 and SB among the 3 chewing rates (p = 0.217, F = 1.594). This suggests that the same amount of stress-loading was obtained under each chewing rate. Salivary cortisol levels in this study after stress loading were greater than the average levels in the same time period in another study. This suggests that stress-loading was successful in this study.

Previous studies have employed various fixed chewing rates irrespective of individual variation. However, varying chewing rate without consideration of individual differences may itself increase stress, thus affecting the results of the experiments. In this study, the fastest, slowest, and mean chewing rates were 1.4, 0.76 and 1.19 ± 0.2 cycles/s, showing definite individual variation. Therefore, in setting chewing rates, we first performed a pilot study to determine a range of chewing rates that would not induce discomfort or masticatory muscle fatigue. The fast rate was defined as being 15% faster than the habitual chewing rate, and the slow rate being 15% slower than the habitual chewing rate.

Although the number of chewing strokes differed among the 3 chewing rates, no significant difference was observed in the integrated EMG of the masseter muscle among the 3 chewing rates. An increase in chewing rate decreases the burst durations of masseter muscle, closely affecting the duration of closing. Compared with slow chewing, fast chewing may have decreased muscle activity per chewing stroke, resulting in no difference in the integrated EMG of masseter muscle over the 10 min chewing period. Chewing rate does not affect the amount of work (integrated EMG of the masseter muscle), but does affect the frequency of movement (number of chewing strokes). Considering the possibility that differences in the number of chewing strokes according to the chewing rate affected stress release, we analyzed the correlation between the number of chewing strokes and changes in salivary cortisol levels (Figs. 6 and 7). A significant negative correlation was found between changes in salivary cortisol levels and the number of chewing strokes from S1 to S2 using Pearson’s correlation coefficient (Fig. 6). There was a weak negative correlation between changes in salivary cortisol levels and the number of chewing strokes from S1 to S3 using Pearson’s correlation coefficient.
coefficient (Fig. 7). These results suggest that differences in the number of chewing strokes within this experimental condition affect stress release.

Conclusion
This study showed that differences in chewing rate affected salivary cortisol levels as a stress indicator of the HPA axis, and suggested that the effect on stress release with fast chewing (15% faster than habitual chewing) is greater than that with slow chewing (15% slower than habitual chewing).

Acknowledgments: We are grateful to the subjects for their kind cooperation in this study. We also thank Dr Mutsumi Takagiwa (Associate Professor, Mathematics Laboratory, Tokyo Dental College) for his help with the statistical analysis. We would also like to thank Associate Professor Jeremy Williams, Tokyo Dental College, for his assistance with the English of the manuscript. Lotte Co., Ltd. (Saitama, Japan) are also acknowledged for their kind gifts of test foods. This work was supported by the grant for the Promotion and Mutual Aid Corporation for Private Schools of Japan.

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