SHORT COMMUNICATION

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Salivary kallikrein-8 as a favorable biomarker for stress response

Salivarni kalikrein-8 kao pogodan biomarker odgovora na stres

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Abstract

Background/Aim. Kallikreins (KLKs) are a group of serine protease enzymes capable of cleaving protein peptide bonds. Besides, they are proteolytic enzymes that mediate the conversion of kininogen (alpha 2-globulin) to bradykinin or kallidin. The aim of the study was to examine whether KLK8 might serve as a novel stress biomarker. **Methods**. Twenty-four students (17 female and 7 male) were included in the study. The general and dental health of the students were evaluated in the appropriate anamnesis format. Unstimulated samples were collected by Sarstedt® saliva collection tubes as recommended: 08.00–09.00 am, 12.00, and 2.00–3.00 pm on the exam day. KLK levels were measured by a KLK8 Human ELISA kit. **Results.** The salivary KLK8 levels in the morning (1.25 ± 0.26 pg/mL) were statistically significantly lower than the KLK8 levels pre-exam [at 12.00 (2.89 ± 0.85 pg/mL)] ($p = 0.0006$). There was also a significant difference in salivary KLK8 levels between pre- and post-exam (1.69 ± 0.39) time points ($p = 0.0005$). **Conclusion.** These results show that the differences in salivary KLK8 levels might be related to the degree of stress, indicating that KLK8 may serve as a novel stress biomarker.

Key words:
biomarkers; kallikreins; oral health; saliva; stress, psychological; students, dental.

Apstrakt

Uvod/Cilj. Kalikreini (KLK) su grupa serin proteaza, enzima koji su sposobni za cepanje peptidnih veza proteina. Pored toga, oni su proteolitički enzimi koji posreduju u konverziji kininogena (alfa 2-globulina) u bradikinin ili kalidin. Cilj rada bio je da se utvrdi da li bi KLK8 mogao biti novi biomarker za stres. **Metode.** Studijom su obuhvaćena ukupno 24 studenta (17 ženskog i 7 muškog pola). Opšte i dentalno zdravlje studenata bilo je procenjivano odgovarajućom anamnezom. Nestimulisani uzorci bili su prikupljani u epruvetama za sakupljanje pljuvačke Sarstedt® na sledeći način: u 08,00–09,00, u 12,00 i u 14,00–15,00 sati na dan ispita. Nivoi KLK mereni su *ELISA* metodom za humani KLK8. **Rezultati.** Jutarnji salivarni nivoi KLK8 (1,25 ± 0,26 pg/mL) bili su statistički značajno niži od nivoa KLK8 pre ispita [u 12,00 sati (2,89 ± 0,85 pg/mL)] ($p = 0,0006$). Takođe postojala je značajna razlika između salivarnih nivoa KLK8 pre i posle ispita (1,69 ± 0,39) ($p = 0,0005$). **Zaključak.** Navedeni rezultati pokazuju da razlike u nivoima salivarnog KLK8 mogu biti povezane sa stepenom stresa, što ukazuje na to da KLK8 može služiti kao novi biomarker stresa.

Ključne reči:
biomarkeri; kalikrein; oralno zdravlje; pljuvačka; stres, psihički; studenti stomatologije.

Introduction

The concept of stress, by definition, refers to the psycho-physiological response of a person to the physiological, mental, and social events that one encounters throughout daily life, which are defined as harmful [1, 2]. In the face of undesirable events, a person can respond to the event with physiological, emotional, cognitive, and behavioral changes. In addition to these adverse effects, stress can also cause severe changes in the hormonal system [3].

Depending on whether acute or chronic stress (CS) forms, changes can occur in the nervous, cardiovascular, endocrine, and immune systems [4, 5]. Although these changes constitute the stress response, they are usually adapted for the short term. In acute stress, glucocorticoids directly inhibit the hypothalamic-pituitary-adrenal (HPA) axis activity, while in CS, steroids can exert direct stimulating effects on the brain. The effects of acute and CS may be independent of each other [6]. Acute stress can trigger allergic reactions such as asthma, eczema, urticaria, gastrointestinal symptoms, high

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blood pressure, pain, and psychotic problems such as panic attacks [7]. CS increases adrenal glucocorticoid levels and can lead to harmful cognitive functions. CS can also cause psychological conditions such as depression and anxiety, muscle and bone problems, sleep disorders, cardiovascular diseases such as hypertension, and metabolic diseases such as obesity and diabetes [2].

The physiological response to stress consists of practical and interconnected systems to maintain bodily integrity, even in the most challenging conditions [8]. One of the most essential complex responses of the organism is the stress response, and this stress response can create adaptive processes [5, 9, 10]. All living organisms must sense and respond to conditions that underlie their homeostatic mechanisms [5, 11]. In addition, severe and prolonged stress responses may activate the nervous system's defense mechanism and damage the organism.

Kallikreins (KLKs) are the human origin family of 15 serine proteases differentiated by their physical properties [12, 13]. They have high substrate specificity defined by their ability to form bradykinin, a vasoactive protein from kininogen [14]. Some are expressed in very few tissues, while others are highly expressed. The protease effect is always unidirectional.

KLKs or KLK-related peptidases catalytic mechanism can be explained by substrate binding, formation of the acyl-enzyme intermediates, transition state stabilization, peptide bond cleavage, release of products, and regeneration of the enzymes that are all facilitated by the active site residues and surrounding protein structure. KLKs are synthesized as inactive zymogens (proenzymes) and are secreted into the extracellular environment. Once activated, they can cleave peptide bonds between the amino acids arginine and lysine in proteins and peptides, thereby exerting their proteolytic activity. This activation can occur through various mechanisms, including proteolytic cleavage by other enzymes or autoactivation [15]. They are expressed in multiple tissues, mainly secreted from the pancreas, salivary glands, pituitary, prostate, and testicles, and take part in several physiological processes. Their primary functions are tissue remodeling and angiogenesis [16], directly related to physical processes such as neurodegeneration [17] and inflammation [18] and others, such as neuronal plasticity, regulation of blood pressure, and electrolyte balance regulation of cell growth and differentiation. Furthermore, recent reports suggest that many other members of this family are related to ovarian and prostate cancers, as well as to diverse diseases of the central nervous system [13, 15].

The basis of salivation is the sympathetic and parasympathetic branches of the autonomic nervous system that innervate the salivary glands. The use of salivary biomarkers to assess stress states in humans has received much attention, as reactions of the sympathetic nervous system (SNS) [19, 20] induce stress markers. Salivary KLKs, a group of substrate-specific serine proteases, are found in secretory granules within salivary gland cells and are secreted into saliva in direct response to glandular sympathetic nerve stimulation and local norepinephrine release [21–27]. Due to this relationship between SNS activity and salivary KLK release, it was thought that the activities of human salivary KLKs might change during the exam stress associated with the response mediated by increased SNS activity [26, 28].

The aim of the study was to determine whether KLK8 would serve as a potential stress biomarker. Still, it was mainly a biological plausibility to hypothesize that level changes could reflect the physiological stress response. Besides, the selection depends on measurability, potential specificity, clinical implications, and previous research findings [29–31]. Salivary KLK levels were determined to confirm the occurrence of a stress response and its severity. Exam stress was evaluated to determine whether sensory afferent activation occurs, and we proposed that KLK8 could be used as a new stress biomarker.

Methods

Subjects

Twenty-four (17 female and 7 male) students with a mean age of 22.5 ± 0.95 years were included in the study. All described protocols were approved by the Ethics Committee of the Gazi University, Faculty of Medicine in Turkey (Ethical Approval No. 831, from November 12, 2018). Informed consent was obtained from all students. The presence of any disease that may affect the HPA axis was accepted as an exclusion criterion.

Saliva sampling

We used Sarstedt® (Sarstedt AG&Co. KG, Germany) saliva tubes explicitly designed to collect and store saliva samples. These tubes were made from a combination of polypropylene (PP) and low-density polyethylene (LD-PE), which provides superior sample quality and quantity performance.

Before collecting unstimulated saliva samples from young adults, they were instructed not to brush their teeth, smoke, eat, or drink anything besides water for at least an hour. Individuals were asked to keep cotton wool in their mouths for one minute to ensure it was completely saturated with saliva.

Biochemical analysis

Saliva was collected from the students in the morning (08.00–09.00 am), before the exam (12.00), and after the exam (2.00–3.00 pm) on the exam day.

Samples were centrifuged at 3,200 × g for 30 min at +4 °C, divided into Eppendorf tubes, and stored at -80 °C until the study was conducted.

Salivary kallikrein-8 concentration

The concentration of KLK8 in saliva (pg/mL) was measured by enzyme-linked immunosorbent assay (ELISA) using a commercial kit KLK8 USCN CEA690Hu Wuhan

USCN Busines Co. Ltd., ELISA sensitivity: 1.73 pg/mL. The intra-assay and inter-assay coefficients of variation were less than 10% and 12%, respectively.

Statistical analysis

Data analysis was performed using the SPSS 18 and Jamovi programs. The means and standard deviations of the samples were used. Parametric one-way ANOVA and Tukey *post hoc* tests evaluated the data comparisons of the groups. Multiple comparisons were conducted using a *post hoc* test to pinpoint which groups contributed to this discrepancy. The values of $p < 0.05$ were considered significant.

Results

The students' mean age and body mass index (BMI) were 22.5 ± 0.95 years and 20.4 ± 0.93, respectively. BMI was calculated for the homogeneity of our group.

Figure 1 indicates the results of the salivary KLK8 levels of the students at different times of the day. The results of each time point in this study suggested differences in KLK8 concentrations in saliva samples which were low during the morning, increased before, and decreased after the exam.

Along with the perception of stress, salivary KLK8 levels were changed between morning, pre-, and post-exam (1.25 ± 0.26 pg/mL, 2.89 ± 0.85 pg/mL, 1.69 ± 0.39 pg/mL, respectively). Higher stress (academic exam) was associated with higher KLK8 levels. With a significance level of $p < 0.001$, it was observed that a notable difference existed between the averages of the groups being compared concerning the examined feature.

When morning salivary KLK8 levels were juxtaposed with pre-exam KLK8 levels, a difference of -1.64 in the averages of these groups was identified. Given a *p*-value of $p < 0.05$, it can be inferred that a significant distinction existed between the averages of the two groups. Notably, the mean KLK8 levels in samples taken before the exam were statistically higher than those collected in the morning. Due to the disparity between the averages in the *post hoc* test, it is evident that a difference existed between the averages of these two groups ($p < 0.001$).

When comparing the salivary pre- and *post*-exam KLK8 levels, the difference between the averages of these groups was found to be 1.20. With a resulting *p*-value of 0.0005, it was determined that there was a significant difference between them.

In the comparison between morning and *post*-exam salivary KLK8 levels, the difference between their averages was found to be -0.44. As the *p*-value was greater than 0.05, no significant difference was found between them. Looking at the average KLK levels in the morning (1.25 pg/mL) and *post*-exam (1.69 pg/mL), it is apparent that the morning levels were statistically lower than those taken before the exam. However, since there was no difference between the averages in the *post hoc* test, it could be inferred that the averages of these two groups are equivalent. Despite their apparent difference, there is no statistically significant distinction between them ($p = 0.164$) (Figure 1).

Discussion

The current study evaluated the differences in saliva KLK8 levels during stress status and suggested that KLK8 might serve as a new, favorable stress biomarker.

The results indicate a potential relationship between stress and salivary KLK8 levels, providing insights into the physiological responses to stress in academic exams.

As a biological fluid that can be analyzed for diagnostic purposes, saliva can be considered a stress-free alternative to blood draw because it is easy, non-invasive, and effortless compared to serum.

KLKs are a subgroup of serine proteases that undertake various physiological functions in human metabolism. Until recently, KLK-1, KLK-2, and KLK-3 were known as human KLKs. However, recent studies have identified up to 12 new

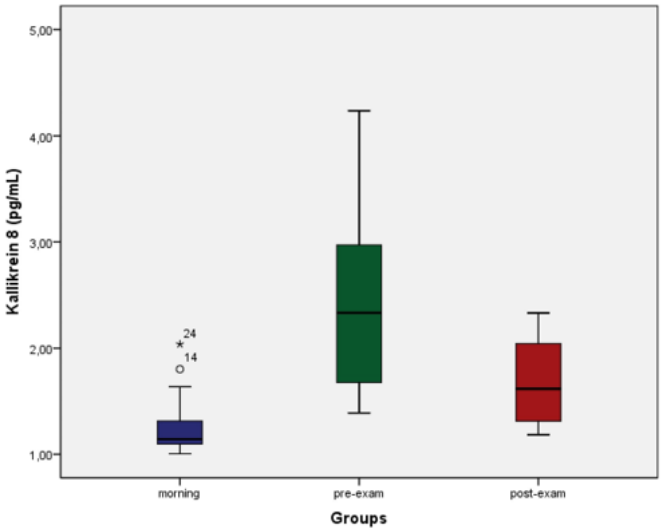

Fig. 1 – Salivary kallikrein concentration was measured at three different times of day.
***p* < 0.001.**
***Note*: numbers 14 and 24 indicate the outliers of sample 14 and 24 within the group.**

members of the KLK family, and some of these new members have been reported to have essential functions in nervous system injuries and diseases [32]. Many studies show that KLKs have the potential to be biomarkers of cancer and neurological diseases.

Based on the provided excerpt, it appears that Smith-Hanrahan [28] conducted a study investigating the association between salivary KLK output and the stress response to surgery. Saliva samples were collected to determine salivary KLK output. These samples were likely obtained using a saliva collection method, such as the passive drool technique or saliva collection devices, both before and several times after surgery. This suggests a longitudinal study design, where samples were collected at multiple time points to track changes in salivary KLK output over time, particularly in response to the stress of surgery. The study's results indicate that increased salivary KLK output is associated with the stress response to surgery [28].

Overall, the study by Smith-Hanrahan [28] suggests that salivary KLK output may be a valuable biomarker for assessing the stress response to surgery. However, it is essential to consider factors such as sample size, study population, and potential confounding variables when interpreting the results. Our findings, which aimed to investigate the effects of stress on salivary KLK8 levels at different intervals, showed that psychological factors (academic exam stress) could influence KLK8 saliva levels. Exam stress is one of the leading causes of emotional stress in students. Therefore, in this study, the salivary level of KLK8 was significantly lower in the pre-exam group than in the *post*-exam group, which indicated the effects of academic stress and was the first study that demonstrated the relationship between KLK8 and stress. The number of exams that young people experience is increasing and overwhelming and hurts mental and physical well-being. The direction of the KLK8 response to a stressor may be related to motivational and emotional factors. However, it is unclear which alterations in saliva mediate the observed change in KLK8.

Salivary biomarkers are well established in psychoneuroimmunology research as measures of stress. Our previous study showed a significant increase in cortisol and amylase levels, known as stress biomarkers, in response to pre- and *post*-exam stress [33]. Our analysis also revealed that the KLK8 level can be used as a new biomarker, as it directly correlates with increased stress. When we look at all these results, saliva sampling plays a potential role for noninvasive, real-time, and point-of-care biomarkers [34]. One of the critical benefits of Sarstedt Salivette® tubes is their ability to perform well with small volumes of samples, which is essential in ensuring that the sample collected is representative of the individual being studied. Additionally, the material used in the tubes is of high quality, ensuring the sample remains stable and protected throughout the collection and storage process. Their high analytical performance makes them a valuable tool in research studies.

Conclusion

Our research findings showed significant changes in KLK8 levels in the morning and before and after the exam, suggesting a relationship between KLK8 and stress response. In addition, this research suggested a possibility of salivary KLK8 being evaluated among stress biomarkers. Therefore, studies aimed at expanding the understanding of the various effects of psychosocial factors and processes on physiological responses to stress can use and benefit from this feasible and noninvasive type of measure. It would be interesting to conduct an additional study regarding this issue in a broader group based on the promising findings presented in this paper. Moreover, further validation studies are necessary to confirm its utility and establish its reliability and sensitivity in assessing stress levels.

Conflict of interest

The authors declare no conflict of interest.

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