

Review article

Biomarkers of Stress in Saliva

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SUMMARY

Stress is defined as a state in which homeostasis, as a dynamic balance of internal conditions necessary for the proper functioning of cells or the living organism as a whole, is affected by the action of various stressors. Stress reaction occurs as a result of stress system activities, which is located in the central and peripheral nervous system. Stress evaluation involves a qualitative and quantitative analyses and valuation of certain biologically active substances (biomarkers of stress) in body fluids that are so often associated with stress. Saliva as a diagnostic medium is being increasingly used for purposes of clinical and basic research because of its composition and content as well as the advantages of the process of sampling, as compared to traditional methods of collecting blood samples and urine samples. Cortisol, as a biomarker of stress, is the most often studied salivary biomarker, which is associated with the activation of the hypothalamic-pituitary-adrenal (HPA) axis. Since stress leads to the suppression of the immune system, values of salivary secretory IgA and salivary lysozyme, as biomarkers of stress, can be analyzed. In saliva, it is difficult to monitor acute stress parameters, catecholamines, due to their low concentrations, rapid degradation and instability in the samples. Chromogranin A (CgA) and α -amylase enzyme can be used as alternative indices of adrenergic activity during stress reactions, due to their stability in saliva and reliability of the obtained values.

Stress reaction and the diseases in whose pathogenesis it participates are yet another proof of the constant interaction of physical, psychological and social factors in health / disease.

Key words: stress, saliva, stress biomarkers

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INTRODUCTION

STRESS

Stress is defined as a state in which homeostasis, as a dynamic balance of internal conditions necessary for the proper functioning of cells or the living organism as a whole, is affected by the action of various factors - stressors (1-3). In such circumstances, homeostasis tends to restore itself with the use of complex mechanisms of physiological and behavioral adaptation of the body to various changes outside or within the body itself. Bodily adaptation is manifested as a synchronized interaction of almost all bodily functions, especially nervous, endocrine and immune systems. Stress reaction mechanisms, as an adaptive force the body generates, are complex. The quality and quantity of bodily adaptation (reaction) to the action of stress stimuli depends on numerous factors: type, intensity and duration of stress, as well as individual characteristics (1-3).

STRESS SYSTEM PHENOMENOLOGY

Stress reaction occurs as a result of stress system activities, which is located in both central and peripheral nervous systems. The system receives and concomitantly processes numerous, various neurosensory (more cortical, limbic, visual, auditory, olfactory, gustative, somatosensory, nociceptive, visceral) impulses as well as signals coming through blood (hormones, cytokines, other mediators) (1-3).

Stress system activation results in a series of temporally limited physiological and behavioral reactions for the purpose of bodily adaptation to the new circumstances the body is now exposed to (1-3). Behavioral adaptation includes heightened excitement, vigilance, caution, focus as well as euphoria or dysphoria. Also heightened is analgesia, increased temperature, with the inhibition of vegetative functions, such as appetite, feeding and reproductive functions. At the same time, physiological adaptation occurs for the purpose of diverting energy, so that oxygen and nutrients are directed towards the CNS and other bodily systems where they are most needed at the moment. When it comes to the cardiovascular system, there is an increase of arterial pressure, minute heart volume, and increased pulse. In

the respiratory system there is bronchodilation and alveolar hyperventilation, and rapid breathing. Also, stimulated are catabolic processes, which results in lipolysis, glycogenolysis, glyconeogenesis, increasing plasma concentration of glucose, fat acids and amino-acids, basic fuels for our body. Also increased is detoxification, for the purpose of ridding the body of damaging metabolism products as a result of stress reactions, while, at the same time, the activity of digestive, reproductive and immune system has been inhibited (1-3).

During stress reactions, mechanisms are activated in the body to inhibit stress reactions. The purpose of these mechanisms is to control stress reactions in terms of preventing excessive responses by stress system components. These mechanisms are essential for successful adaptation. Without them, stress reaction becomes oversized, prolonged, going beyond the framework of adaptation, which may result in the development of certain pathological conditions. The mechanisms that stimulate and the mechanisms that inhibit the development of a stress reaction are in constant dynamic balance. Prevailing during reactions to a fight or flight is the activity of those mechanisms that stimulate the development of a stress reaction, while prevailing during the process of recovery from stress is the activity of those mechanisms that inhibit the development of a stress reaction. It is very important to have a timely appeasement or neutralization of effects started by the action of stress stimulus as soon as immediate danger passes, since the stress reaction outside the period of action of stress stimulus is rather exhausting for the body (1-3). After the stress reaction, the period dominated by catabolic processes (energy mobilization) as a result of stress hormone effect (catecholamine and cortisol), it is necessary to have a period of rest and recovery. The period of rest and recovery is supposed to provide a more intensive activity of anabolic processes based on the activity of growth hormone and gonadal steroids, which are in charge of recovery, healing and growth. The balance between catabolic and anabolic processes is but a segment of homeostatic balance each cell strives to, as well as the body as a whole (1-3).

It should be borne in mind that each state of stress is not "a priori" damaging for the body. It has been opined that mild, brief and controlled states with which to "condition" homeostasis may be accepted as a pleasant, exciting and certainly encouraging

stimulation for an individual's emotional and intellectual growth and development (2).

PHYSIOLOGICAL ASPECTS OF THE STRESS SYSTEM

Sympathetic system in a stress reaction

The autonomous nervous system controls the function of internal organs and organic systems through the sympathetic and parasympathetic nervous systems. Thanks to its effect on the adrenal medulla, the sympathetic system provides for the release of catecholamine directly into circulation, which has significance during stress reaction as a form of quick response to the stressor effect (1-3). The acute form of stress reaction is a reaction by the type of fight or flight and is based on the registration of unpleasant stimulus (alarm) which is transferred from the cerebral cortex to the nucleus of LC/NE brain stem and pons, whose neurons, via relevant receptors, stimulate the release of catecholamine (adrenalin and noradrenalin) from the adrenal medulla. Catecholamines make the body more alert and cautious, they activate defense behavior patterns, with increased aggressiveness, and have a stimulating effect on the cardiovascular and respiratory systems, with the inhibition of the gastrointestinal tract function, which allows for the re-distribution of blood from the gastrointestinal system to the muscular system and brain. The increased amount of catecholamine in stress situations stimulate lipolysis and glycogenolysis, thus increasing plasma concentrations of glucose and fat acids, basic fuels for the body. These types of reactions are significant for the purpose of survival and protection of the body from various physical factors that represent a risk for the body as a whole (1-3).

The hypothalamic-pituitary-adrenal axis in a stress reaction

A somewhat slower, second response during a stress reaction is an increased activity of the so-called HPA (hypothalamic-pituitary-adrenal) axis. On the hypothalamic level, the stressors activate the secretion of the CRH and AVP as a synergist with the CRH (1-4). The CRH and AVP secretion to pituitary blood

vessels brings about the release of ACTH from the pituitary gland frontal lobe into peripheral circulation. Through circulation, the ACTH travels to adrenal cells where it stimulates the synthesis and secretion of glucocorticoids (1-4). Glucocorticoids are steroids by nature, they are created from cholesterol and make their effect through specific receptors located in the cytoplasm of target cells. Thus created receptor-hormone complex enters the cell nucleus where it activates the transcription of specific genes for the creation of mRNA. The mRNA created this way goes to cytoplasm and in the ribosomes stimulates the process translation for the creation of new proteins. The newly-created proteins act as enzymes or transport proteins that modulate and/or activate some other cellular processes (4).

Cortisol, CRH, AVP, ACTH are all subject to the circadian rhythm of secretion, with the highest concentration in tissue fluids being achieved in the morning hours, with a tendency of decline during the day, so that the lowest values are reported in the evening hours. Secretion is significantly increased during the states of stress (4).

The ultimate effect of the HPA axis activation is the domination of catabolic processes in the body, in order to render available to the body enough energy substrates, with the aim of meeting the increased need of the body at a moment important for its survival. The high plasma concentration of cortisol stimulates gluconeogenesis in the liver and produces insulin resistance of peripheral tissues, while the increased secretion of adrenalin and noradrenalin stimulates glycogenolysis and lipolysis (1-4). Glucocorticoids play a rather important role in controlling the stress response duration, the negative feedback mechanism, which aims to minimize catabolic, lipolytic, anti-reproductive and immunosuppressive effects of stress reactions (1-4).

METHODOLOGICAL APPROACHES TO STUDYING STRESS

The available information from literature confirms that two different methodological approaches were mostly used during the studies of stress and stress reactions. One group of authors have been studying stress in laboratory conditions, using the standardized method Trier Social Stress Test (TSST) (5). The test includes a time-limited public

exposure and solving mathematic problems before listeners. The other group of authors have opted for studying stress in natural conditions, meaning the studying of stress as a result of stressor action in everyday life, so-called "real-life" stressors (6). Dickerson and Kemeny (7) have shown that the efficiency of stressors in laboratory conditions depends on the combination of different psychological factors affecting the subject, as a result of interaction between structural traits of a person (ego), his assessment of the degree of social vulnerability, and his assessment of loss of control over the given situation during the stressor exposure. Naturally, these psychological factors also have significance in studying stress in natural circumstances. This method, compared to laboratory conditions, has greater validity, but at the same time carries a problem of inability to standardize, which results in the lack of homogeneity of the obtained results (8). Taking academic exams in the student population (6, 8, 9) and the stress related to a particular profession are frequently used methodologies in the evaluation of stress as a result of "real-life" stressor action (10, 11).

Stress evaluation includes quality and quantity assessment of certain biologically active components (biomarkers of stress) in bodily fluids that may be related to stress.

SALIVA AS A DIAGNOSTIC MEDIUM

Saliva, as a product of large and small salivary glands in the mouth, has multiple functions. Firstly, saliva is necessary in the process of chewing and swallowing food. Its role in the digestive process is reflected in the activity of amylases, the enzymes that participate in the food digestion process. Saliva also has a defensive function, contains secretory IgA that is involved in the immune response, while, on the other hand, antibacterial effect is made thanks to the presence of peptides (lysozyme) that inhibit mouth flora microorganisms. Also, saliva contains a broad range of signal molecules: hormones (steroids, peptides, amines), enzymes (lysozyme, α -amylase), immunoglobulin (IgA) and other proteins (e.g. Eosinophil Cationic Protein) and DNA, which find their way into saliva from blood vessels or as direct products of salivary glands, manifesting their effects on target cells (12, 13).

As of recently, the results of numerous studies suggest an increasing number of possibilities to use saliva as a diagnostic medium, for the purpose of clinical and basic research. Saliva is increasingly used in the qualitative and quantitative analysis of biologically active components, as an alternative to traditional blood and urine sampling methods. Saliva sampling methods, as compared to blood sampling, are less invasive, simpler, safer, less stressful, easy to repeat, do not require any particular training and equipment, and are thus becoming ever more acceptable, and reasons for their use ever more justifiable (14).

Naturally, the collection, storing and preparation of samples, as well as the very protocols for analysis vary, depending on which component is being analyzed. Various techniques are used for the purpose of taking saliva samples. It is possible to take a sample of a particular salivary gland (by suction techniques or by inserting the cannula into the salivary gland channels) as well as mixed saliva samples. These methods are simpler, mostly routine, and require no hospital setting. Also, the direct mixed saliva sampling method implies spitting, as well as spontaneous dribbling of saliva over the edge of lips into the collecting vessel, while indirect methods, which are considered more comfortable for the subject, imply the use of absorbent tissues in the mouth, from which saliva is separated by centrifugation (14).

Immunological (RIA – Radioimmunoassay; ELISA – Enzyme-linked immunosorbent assay) and chromatographic methods are most frequently used analytical methods in the salivary component analysis due to high sensitivity and specificity (14).

SALIVARY BIOMARKERS OF STRESS

Results of numerous stress studies have pointed to their correlation with several salivary biomarkers of stress as objective indicators of stress reactions. The level of salivary cortisol is thus brought into relation with the activation of the hypothalamic-pituitary-adrenal (HPA) axis (4,7,9,14,15). Since stress shows the immunosuppressive effect, the level of secretory IgA (10,11,15) and lysozyme (16) in the saliva is, also, in the available literature, used as an objective stress reaction biomarker. The activation of the sympatho-adrenomedullary system results in the

release of catecholamine, which is difficult to track in the saliva because of low concentration, rapid degradation and hormone instability in the samples taken. It is also possible to use chromogranin A (CgA) (14, 17) and enzyme α -amylase (14, 18) as a response of the sympatho-adrenomedullary system to stress, or as biomarkers of stress, as alternative indices of adrenergic activity, due to their stability in the saliva and reliability of the obtained values.

Cortisol

Cortisol, as a biomarker of stress, is the most frequently studied substance in the saliva (4, 9, 14, 15). Salivary cortisol is present in a free, unbound form and in correlation with unbound blood plasma cortisol, the only hormone fraction that shows metabolic activity. In blood plasma, most of the cortisol (65%) is bound with high affinity and low capacity to the corticosteroid-binding globulin (transcortin). A total of 30% of cortisol is bound to albumins, while 3-5% of cortisol is in an unbound, free form whose blood analysis is demanding, time-consuming, and not suitable for the routine, clinical practice (4, 7, 14). Salivary cortisol concentration accounts for 70% of unbound blood cortisol which, due to its low molecular weight and liposolubility, by way of free diffusion, passes through the basal membrane of acinar cells of salivary glands into saliva. Cortisol is metabolized in the liver, 25% is secreted through bile, while the remaining 75% is secreted through kidneys in a free form. However, renal secretion depends on glomerular and tubular function, while the rate of cortisol secreted daily via urine depends on the correctness of the procedure of collecting urine over the 24 hours. Urinary cortisol is not always correlated to the free cortisol blood concentration, partly for the foregoing reasons, while the other reason is that during renal filtration cortisol is converted into hydrosoluble metabolites, which is not the case with salivary cortisol. In line with the mentioned facts, the results of numerous studies have confirmed that the analysis of salivary cortisol as a biomarker of psychological stress is a reliable alternative to blood and urinalyses (4, 7, 14).

Balance between blood and saliva cortisol is achieved in less than 5 min regardless of the saliva flow (7, 14). The highest cortisol concentration is reported in the morning hours, 45-60 minutes after

waking up, while the lowest concentration is reported in the evening hours (7), which is in correlation with daily variations of plasma cortisol. Research results have shown that peak levels of cortisol are detected in blood 30 minutes after exposure to stressors, while 41-60 minutes after cessation of stressor action the value of total cortisol returns to the values before the stressor action (7).

Immunoglobulins

The relation between mental stress, HPA endocrine system and immunity is a complicated one. Psychological stressors increase the level of cortisol through the increased activity of the adrenal gland. The increased level of cortisol inhibits the functions of lymphocytes, macrophages and monocytes (1-3).

Secretory IgA is the dominant antibody in saliva, which is, thanks to its structure, resistant to the effect of proteolytic enzymes. Daily secretion is 66 mg/kg/day, while the average half-life (elimination) of secretory IgA ranges from 3 to 6 days. The protective effect of secretory IgA depends on the secretory IgA concentration in saliva, as well as on the saliva flow. Results of the studies conducted on subjects aged 20-45 show that secretory IgA in saliva peaks during the initial 30 minutes after waking up, and then declines gradually over the following 4 hours, after which it reaches plateau for the rest of the day (15). Also, Clow *et al.* (15) have shown that daily variation of secretory IgA concentration in saliva is closely related to daily variations of salivary cortisol. However, it is still unclear whether the daily variations of secretory IgA in saliva are a response to cortisol secretion or whether they are controlled by other mechanisms.

Secretory IgA in saliva has been used as a stress marker in several studies, but the results obtained are contradictory. On the one hand, it was established that secretory IgA in saliva stands in a negative correlation to the level of the experienced stress. By the use of adequate psychological instruments, standardized scales (The Perceived Stress Scale – PSS), the subject was enabled to evaluate the experienced stress by himself. Consequently, a cross-sectional study encompassing 106 emergency nurses and 56 general nurses, where the mentioned stress scale was applied and concentration of secretory IgA in saliva measured, has shown that emergency nurses experience greater stress (on average 1.51 vs. 1.30) and

at the same time have significantly lower values of secretory IgA in saliva (49.1 vs. 68.2 $\mu\text{g}/\text{min}$) in relation to general nurses (10). It was also established that the value of secretory IgA in saliva stands in negative correlation to the degree of stress experienced among the tested populations of dental students (students on undergraduate and postgraduate studies) (Spearman's $r = -0.20$, $p < 0.05$) (19). On the other hand, Ng *et al.* (20) have studied differences in the experience of stress in 31 junior year dental students, before and after exams, measuring the levels of cortisol, secretory IgA and chromogranin A (CgA) in saliva. The results indicate that a higher stress score was reported before the exam in relation to the values taken after the exam ($p = 0.015$), that cortisol levels were higher before the exam ($p = 0.015$), while a difference concerning the values of secretory IgA and CgA in saliva before and after the exam was not statistically confirmed.

Lysozyme

Lysozyme is a low molecular mass cationic protein, widely present in tissues and tissue secretions. Its synthesis takes place in monocytes and macrophage from where it is constantly released. Lysozyme shows its anti-bacterial effects by hydrolyzing the bond between N-acetylmuramic acid and N-acetylglucosamine, components of the bacterial cell wall.

Perera *et al.* (16) have studied the impact of stress on congenital mucus membrane immunity, by measuring the values of salivary lysozyme among a population of students (39 students) who took exams at the end of the school year. The obtained values of salivary lysozyme correlate negatively ($r = -.477$, $p < .01$) to the level of experienced stress (stress level assessed by the use of standardized stress scale). Lysozyme concentration was significantly ($p < 0.05$) lower in the saliva sample taken before the exam in relation to the values obtained after the completion of all exams. Yang *et al.* (17) have shown that the value of salivary lysozymes is related to mental health, which means that the value of salivary lysozyme stands in negative correlation to the level of experienced professional stress in emergency nurses in relation to general nurses.

Chromogranin A

Chromogranin A is an acidic protein present in secretory granules of various endocrine and neuroendocrine cells. It has been opined that it operates as a pro-hormone with multiple meanings in secretory processes; however, the exact role of these proteins has yet to be fully clarified. Chromogranin A (CgA) is stored in the cells of adrenal gland core, and is released from vesicles by the process of exocytose together with catecholamines. Immunohistochemical analyses have confirmed its presence also in submandibular glands (21). During their research, Giampaolo *et al.* (22) have demonstrated a daily CgA rhythm in healthy subjects, where peak levels are reached during the night (23:00 h) and minimum levels in the morning hours (08:00 h). Average half-life (elimination) of CgA in blood is 18.4 min, while correlation between the blood and saliva values has yet to be confirmed with certainty.

Results of the studies pertaining to the impact of acute stress on the salivary CgA level are contradictory. Takatsuji *et al.* (18) have looked into the effect of tested stress on the salivary cortisol, secretory IgA and chromogranin A (CgA) levels in 15 female students. Saliva samples were taken immediately before the exam, right after the exam, which lasted for 1h, as well as 2h after the exam. Results of the analysis have shown that the values of secretory IgA and CgA were statistically significantly increased right after the exam and reduced two hours after the exam, while statistical significance was not confirmed for salivary cortisol values.

However, on the other hand, we have the already mentioned studies by Ng *et al.*, (20) whose objective was to monitor the difference between the experience of stress in 31 junior year dental students, before and after the exam, by measuring the levels of cortisol, secretory IgA and CgA in the saliva. The obtained results indicate that a higher stress score was reported before the exam in relation to the values after the exam ($p = 0.015$) (with the use of standardized tests for stress self-assessment), then also that the cortisol level was higher before the exam ($p = 0.015$), while, statistically, the difference between values of secretory IgA and CgA in the saliva before and after the exam was not confirmed.

α -amylase

Salivary glands excrete the enzyme α -amylase as a result of adrenergic stimulation. As for the daily rhythm, the lowest level of α -amylase in saliva was reported in the morning hours, and the highest in the late afternoon, in contrast to cortisol and salivary IgA. Half-life (elimination) of α -amylase in plasma is about 12-24h, while values for saliva have yet to be determined with certainty (23).

In the available literature, the results pertaining to α -amylase as a possible biomarker of acute stress are quite balanced. Strahler *et al.* (24) have looked into the level of salivary α -amylase in 3 population groups of various age (children, younger adults and older adults) following their exposure to stress in laboratory settings (the use of standardized TSST protocol). The values of salivary α -amylase increased rapidly as a response to the psychological stress in all three tested groups. Also, Schoofs and Wolf (25) have analyzed in laboratory settings (TSST) the effect of stress on the level of salivary cortisol and α -amylase as objective markers of HPA axis activity, or sympatho-adrenomedullary system in 39 young men and 44 women. The measuring of salivary parameters was carried out using saliva samples, taken immediately before, 10 min and 25 min after exposure to stress, as prescribed by the protocol. The values of both

parameters increased as a response to stress. The results unequivocally show that α -amylase is sensitive to stress.

CONCLUSION

In a modern society, stress and stress reaction have a decreasingly protective character aimed at helping the body to cope with the given situation, and an increasingly damaging effect on the body, going beyond the boundaries of coping.

Stress reactions and diseases in whose etiopathogenesis they participate are yet another proof of continual interaction between physical, psychological and social factors in health/disease, and the justifiability and necessity of a multi-disciplinary approach to the evaluation and treatment of certain conditions.

Studies conducted in the field of stress and stress reactions have certainly been upgraded by the use of specific quantitative and qualitative salivary analyses. The removal of noticed deficiencies, as well as a standardization of collection and analysis procedures, will in the near future make possible for saliva to be accepted as a reliable, equal, additional diagnostic medium with a significantly larger presence in everyday clinical practice.

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Biomarkeri stresa u pljuvački

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SAŽETAK

Stres se definiše kao stanje u kome je homeostaza, kao dinamička ravnoteža unutrašnjih uslova neophodnih za pravilno funkcionisanje ćelija ili živog organizma u cjelini, ugrožena djelovanjem različitih stresora. Stresna reakcija nastaje kao posljedica aktivnosti tzv. stres sistema, koji je smješten u centralnom i perifernom nervnom sistemu. Stres sistem, preko odgovarajućih medijatora, stimuliše kataboličke, lipolitičke, antireproduktivne i imunosupresivne efekte stresne reakcije s ciljem preusmjerenja energije zbog povećane potrebe organizma u trenutku značajnim za njegovo preživljavanje. Evaluacija stresa podrazumijeva kvalitativno i kvantitativno analiziranje i vrednovanje određenih biološki aktivnih komponenti (biomarkera stresa) u tjelesnim tečnostima koje se dovode u vezu sa stresom. Pljuvačka se kao dijagnostički medijum sve češće koristi za klinička i bazična istraživanja zbog mogućnosti koje pruža, s obzirom na njen sastav i sadržaj kao i na prednosti samog procesa uzorkovanja u odnosu na tradicionalne metode prikupljanja uzoraka krvi i urina.

Od biomarkera stresa, u pljuvački je najčešće ispitivan kortizol, koji se dovodi u vezu sa aktivacijom hipotalamus-hipofiza-adrenalne (HPA) osovine. S obzirom da stres dovodi do supresije imunog sistema, u pljuvački se mogu analizirati i vrijednosti sekretornog IgA i lizozima kao biomarkera stresa. Parametre akutnog stresa, kateholamine, teško je pratiti u pljuvački zbog njihove male koncentracije, brze degradacije i nestabilnosti u uzetim uzorcima. Kao alternativni indeksi adrenergičke aktivnosti tokom stresne reakcije, zbog stabilnosti u pljuvački i pouzdanosti dobijenih vrijednosti, mogu se koristiti hromogramin A (CgA) i enzim α -amilaza.

Stresna reakcija i oboljenja u čijoj etiopatogenezi ona učestvuje još su jedan dokaz stalne interakcije fizičkih, psihičkih i socijalnih faktora u zdravlju/bolesti.

Ključne reči: stres, pljuvačka, biomarkeri stresa