State of the Science: Salivary Biomarker Utilization for Stress Research

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Purpose: The use of salivary biomarkers for stress research is increasing based on the convenience of collection, affordability and scientific merit. This short review provides an overview of the state of the science of salivary biomarkers utilized in research related to stress.

Methods: An integrative review was conducted.

Results: The trend of utilizing salivary biomarkers in stress research was reviewed, specifically, focusing on the use of endocrine and inflammatory biomarkers incorporated in previous stress research. Then, a review of sampling procedures for salivary biomarkers and the analytic methods is provided. Finally, a discussion on the strengths and areas for improvement in the use of salivary biomarkers in stress research is included.

Conclusion: Salivary biomarkers as an alternative to blood biomarkers are increasingly being recognized as a legitimate source for analyzing the stress response in humans.

Key Words: Stress, Saliva, Biological marker, Cortisol

INTRODUCTION

A biomarker is defined as “a characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention”.¹ In the last two decades, the term “salivary biomarker” has been used to indicate the biomarkers detected in saliva and related publications have increased dramatically. A literature search on PubMed using the keywords “biomarkers and stress” identified 11,612 results between 2004 and 2014, whereas “salivary biomarkers and stress” identified 302 related publications, a small portion of the body of research devoted to biomarkers and stress.² Despite the increased use of saliva for measuring biomarkers, the number of published articles using salivary biomarkers in stress research still accounts for less than 5% of the total number of publications in this field. This suggests that salivary biomarkers compared to traditional biomarkers are still underutilized in stress research.

The purpose of this paper is to provide an overview of the current state of the science regarding salivary biomarkers for stress research. We will first review the trends of utilizing salivary biomarkers in stress research specifically, we focus on the use of endocrine and inflammatory biomarkers incorporated in previous stress research. Next, a review of sampling procedures for salivary biomarkers and the analytic methods will be provided for future researchers. Finally, a discussion on the strengths and areas for improvement in the use of salivary biomarkers in stress research is provided.

SUBJECT

1. Trends of Utilizing Salivary Biomarkers in Stress Research

The term “biomarker” dates back as early as 1980.³ The number of laboratories and grants funded specifically designated to conduct research on biomarkers show the rapid growth in the volume of research supported by the National Institute of Health (NIH) of the states and the European Research Council (ERC) or Cancer Research UK in Europe.⁴,⁵ Biomarkers have
been detected predominantly in blood samples and extensively used in clinical trials for their diagnostic value. With the advancement of biomedical science, assays that are designed specifically for saliva samples have become available, and subsequently, many biomarkers that once required blood samples have been validated in saliva.

Saliva is a clear, slightly acidic (pH=6.0~7.0) and protein-rich fluid composed of secretions from major salivary glands: the parotid, submandibular, and sublingual glands. In general, human salivary glands produce about 1~1.5 liter of serous and mucinous saliva daily. When pathologic health conditions arise (e.g., cancer, inflammation), proteins and substances linked to the disease are transferred to the saliva. Increased concentrations of these compounds over time make saliva a diagnostic fluid with numerous advantages over blood. The advantages can be summarized below:

1) Bypassing often painful invasive procedures

Saliva biomarkers require only non-invasive saliva collection, offers unique opportunities to bypass often painful invasive procedures, such as biopsy and repeated blood draws that add enormous stress to the individuals who already go through unpleasant experience related to their health conditions. Saliva is easy to collect without painful needle sticks, and can be tested in many non-clinical settings for field studies because of portability.

2) Economic consideration

Salivary biomarkers have economic advantages over blood samples with lower costs to store and process samples, perform tests, and less time for doctor or hospital visits to collect samples, thus improving patient satisfaction.

3) Diagnostic utility

Use of salivary biomarkers as a diagnostic tool at the point of care has been tested for detection of numerous conditions and diseases, such as acute myocardial infarction, oral cancer, pancreatic cancer, cardiovascular disease, human immunodeficiency virus (HIV) status and periodontal disease.

4) Scientific merits and convenience for conducting research

Salivary biomarkers offer scientific merits and convenience for conducting research, particularly studies on the physiological mechanisms of the stress response.

Monitoring salivary biomarkers provides a sound, cost-effective and relatively simple measure of stress response because (a) stability is a great asset of salivary biomarker measurements that allows researchers to monitor biomarkers repeatedly for longitudinal studies, (b) low cost and convenience of the sampling procedure of saliva offers an alternative to blood biomarkers, particularly for large population studies, (c) the non-invasive sampling dramatically reduce disturbance related to sampling procedure compared to the conventional blood draw to the research participants, thus minimizing chances of confounding major variables of interest in stress research. With the growing number of studies on salivary biomarkers, the potential for using salivary biomarkers as diagnostic and research tools deserves more attention from the health science and research communities.

2. Utilization of Salivary Endocrinal and Inflammatory Biomarkers in Stress Research

Because the ultimate interest of stress research is to understand, predict and modify the impact of stress on human health and behavior, monitoring biomarkers is imperative for understanding the physiologic responses to stress and their impact on human health. Saliva contains a wide range of biomarkers that reflect physiologic responses to perceived stress. Among these, endocrinal and immune markers—such as cortisol and cytokines, respectively—have been extensively used in stress research. For example, the link between psychosocial stress and development of cardiovascular disease has provided a model of stress-health impact that involves the cascading responses of neuro-endocrinal and immunological responses to stress.

The stress response is regulated by two primary neuroendocrine systems: the hypothalamus-pituitary-adrenocortical (HPA) axis and sympathetic adrenomedullary (SAM) systems. Psychological stress increases HPA activity and, subsequently, elevates the level of cortisol in circulation. On the other hand, the primary mechanism of defense against stressful stimuli is activation of the SAM system, comprising the sympathetic nervous system.
been well established in human stress research. 12,29,33) Cortisol is the most frequently measured salivary biomarker in stress research. A wide range of salivary biomarkers have been identified and tested for their diagnostic value, and still new biomarkers are being identified and validated. They include dehydroepiandrosterone (DHEA) testosteron, and estradiol, Salivary biomarkers also include toxins, metabolites, enzymes (α-amylase, or α-amylase), immunoglobulins (IgA), proteins and DNA. Saliva contains the insulin sensitivity marker, adiponectin cardiac enzymes such as creatinine kinase (CK), myoglobin (MB), and creatine phosphokinase (CPK) as well as neuroendocrine hormones such as epinephrine, norepinephrine, and DHEA. In the following section, we will review three major neuroendocrine markers (cortisol, α-amylase, and DHEA) and inflammatory cytokines that are often measured in stress research.

1) Salivary cortisol

The use of cortisol as a marker for HPA axis activity has been well established in human stress research.12,29,33) Salivary cortisol has a diurnal peak of 13.8~48.9 nmol/l compared to blood, 190~690 nmol/l. Since the diurnal rhythm of cortisol release is well known, cortisol is usually measured in a time series, with few exceptions to reflect the awakening stress response, recovery from awakening, as well as its changes throughout the day until bedtime.12,39) Normal salivary cortisol level varies depending on the studies: 0.67±0.12 to 15.5±0.8 nmol/L (range 10.2~27.3) at 8:00 AM.33) The most frequently used protocol is the one described by Pariante et al., (2004)40) that used the total salivary cortisol output, calculated as the area under the curve (AUC) during the day (MN, 8 AM, and 8 PM) and the AUC of the increase (AUG) of cortisol level after awakening (from 0 to 15, 30, 60 minutes after awakening). The amount of bioavailable cortisol in saliva assessed by the immune assays reflects the HPA axis activity which is of major interest in both research and clinical diagnosis.40) Also, besides immunoassay, ultra-sensitive nano-tube immuno-sensor is available for a rapid measurement of salivary cortisol.

2) Salivary α (α)-amylase

The salivary enzyme α-amylase is secreted by the parotid gland, Properties of α-amylase have been extensively studied in the last decade, While cortisol reflects the HPA axis activity, α-amylase has been used as a novel biomarker of SAM activity.25,42) The potential of salivary α-amylase as a salivary biomarker of adrenergic activity is of interest because it allows the parallel investigation of the two major neuroendocrine response systems—HPA and SAM axis—using non-invasive salivary samples. Salivary α-amylase levels have been shown to increase in response to both physical and psychological stressors, and offers a non-invasive measure for research on stress responses.42,43) Secretion of α-amylase is increased by acute experimental stressors, but is reduced by chronic stress. α-amylase has an endogenous diurnal rhythm like cortisol, thus it may not be reliable marker of catecholamine levels. Another asset of saliva α-amylase is its stability, α-amylase activity is stable over 21 days up to 37°C, and in the absence of bacterial contamination, bacterial growth is limited. These properties make α-amylase activity useful as a biomarker of stress-related autonomic activity that complements plasma catecholamine and cardiac responses to psychosocial stress.25) Stress-related increases in salivary α-amylase can be inhibited by administration of the commonly used adrenergic blocker, propranolol,43) but the β-adrenergic agonist stimulates salivary α-amylase release.44) These properties provide investigators with an important methodological caution to avoid those who use prescription medications for angina or high blood pressure that contain a β-blocker or similar actions in biobehavioral research using salivary α-amylase measurements.

The pattern of stress-related change in salivary cortisol and α-amylase levels are different from each other.12,45) Both salivary analytics increase in response to psychosocial stress, however, the level of salivary α-amylase reaches the peak faster, then, returns to the pre-stress level faster than the level of salivary cortisol. This difference is consistent with the physiological differences of the sympathetic nervous system (SNS) response (quicker) and HPA (slower) response to stress. The differences may be due to the higher sensitivity to stress in SNS than in HPA axis.33) While cortisol and α-amylase reflect different stress mechanisms, they coordinate the stress response to influence cardiovascular health outcomes.

3) Dehydroepiandrosterone (DHEA)

DHEA is a steroid hormone that is produced in the adrenal cortex and can be found in saliva, DHEA has
shown inverse correlation with cortisol levels, indicating protective effects against stress.\textsuperscript{34,35} Shirotuki (2009) reported that complete HPA axis reactivity to acute psychosocial stressors is blunted in anxious individuals as evidenced by a lower cortisol to DHEA ratio in response to psychosocial stress. This finding suggests that salivary level of DHEA, in addition to cortisol, provides a valuable tool, therefore, should be should be considered when investigating biomarkers of stress in this population.\textsuperscript{34}

4) Inflammatory markers
A wide range of inflammatory biomarkers has been used for stress research that include interleukin 1 (IL-1), IL-6, C-reactive protein (CRP), and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)).\textsuperscript{15,46} Inflammatory biomarkers have contributed to advancing our understanding of the role of inflammation in stress physiology.\textsuperscript{12,30} The HPA axis and autonomic nervous system responses to stress manifest in lowering immune function as shown by numerous studies for the last three decades. Chronic psychosocial stress, such as job strain, low socioeconomic status, and caregiver stress are related to the impairments of immune function,\textsuperscript{12,20} which are linked to the development of health conditions such as atherosclerosis,\textsuperscript{21,26,30,47} and cancer.\textsuperscript{15,16} Salivary immunoglobulin A (IgA) and lysozyme were inversely correlated with self-reported level of stress, suggesting their utility as potential stress biomarkers.\textsuperscript{35} Studies to identify and validate new salivary biomarkers for stress research is ongoing.

3. Saliva Sample Collection, Storage, Centrifugation and Time of Measurements

Methods for saliva collection have significantly advanced over the last decade, leading to standard and reliable methods, with devices and techniques that produce consistent salivary samples that provide the most accurate results\textsuperscript{10,13}.

For salivary sample collection, two standard methods have been used: cotton roll and passive drool. In a review of prior studies incorporating salivary biomarkers, some studies describe whether they used cotton roll or passive drool,\textsuperscript{10,22} but most did not specify the method used to collect saliva samples. Saliva samples must be collected in a protease free tubes to optimize stability for a long term storage. After collecting saliva samples, storage conditions and the centrifugation process before assay are similar yet inconsistently reported across studies. Saliva samples are stored in -18, -20, or -80°C freezer until assay.\textsuperscript{47} Centrifugation was at 3,000~3,500 rpm and the duration of centrifugation varied between 3 minutes\textsuperscript{47} and 10 minutes.\textsuperscript{20}

Some studies described sampling time to capture the peak level of cortisol or when the diurnal rhythm was considered to affect the biomarkers of interest.\textsuperscript{48} For example, the level of cortisol was measured between directly after awakening and 20 or 30 minutes after awakening to capture the peak level,\textsuperscript{20,47} then, saliva samples were collected several times during the day until evening and at bedtime\textsuperscript{20,21,47} to investigate the diurnal rhythm of cortisol. When urine samples were collected along with saliva, similar time intervals were used for levels of cortisol as well as epinephrine and norepinephrine.\textsuperscript{20} When the time series was not chosen, sampling time for saliva cortisol was usually in the morning.\textsuperscript{49}

4. Issues related to Use of Salivary Biomarkers

Much attention has been paid to improving the measurement of salivary biomarkers, mainly due to its nature of non-invasive sampling that gives much convenience to both researchers and participants.\textsuperscript{27} We will discuss some concerns and its remediation below.

1) Reaching optimum reliability and validity
For optimum reliability and validity, saliva sampling, storage and analysis have to strictly follow standard procedures (see description above). In studies that require research participants to collect saliva by themselves, detailed guidance should be provided with demonstration and even with practice, if necessary. To conquer this concern, our recent study has been successful in providing guidance by using a video clip that is stored on the participant’s mobile device which provides details for the sampling procedures of saliva.\textsuperscript{50}

2) Difficulty in some populations
In very young children, older or frail populations, collecting saliva can be unexpectedly difficult, time consuming, and may not yield a sufficient volume of specimen for assay. Granger and colleagues (2007)\textsuperscript{10} provided a list of these pitfalls and made suggestions for future researchers. Challenges in saliva collection from full-term and preterm newborns, and infants less than 3 months of age has been well documented.\textsuperscript{51} More time is needed to collect sufficient volume of saliva for assays because newborn infant’s parotid glands have low fluid production rates compared to older children and adults. On the opposite end, collecting saliva samples from the
comes out of this biomarker enterprise. 4) Likewise, new validation will be the key for clinically meaningful out-
nologies and improving productivity in biomarker vali-
As Poste (2012) proposed, connecting these new tech-
complexity of biomarker validation will be increasing.
identified and validated and the technical and logistical
sequencing for clinical use, new biomarkers will be
of rapid expansion of whole exome and whole genome
profile a large number of analytes in a single assay using
platforms. Together with the pending prospect
of diagnostic components as blood samples, there have been
concerns that the low concentration compared with lev-
els in the blood may prevent salivary diagnostics from
being clinically useful. However, with the development
of new and highly sensitive techniques (e.g., molecular
diagnostics, nanotechnology), the low concentration of
analytes in saliva is no longer a limitation. 9,14,25,53,54) To
date, molecular and proteomic technology yield ad-
vanced insight into the characteristics of salivary pro-
tomes and provide strong evidence supporting the use
of saliva as a diagnostic tool. 9,17,25,53-56)

In sum, biomarker research is advancing the ability to
profile a large number of analytes in a single assay using
high throughput genomics, proteomics, 17) and other
‘omics’ platforms. Together with the pending prospect of
rapid expansion of whole exome and whole genome
sequencing for clinical use, new biomarkers will be
identified and validated and the technical and logistical
complexity of biomarker validation will be increasing.
As Poste (2012) proposed, connecting these new tech-
nologies and improving productivity in biomarker vali-
dation will be the key for clinically meaningful out-
comes out of this biomarker enterprise. 40 Likewise, new
salivary biomarkers should be identified and validated
for improved patient management and clinical outcomes
for those who suffer diverse forms of stress-related health
issues.

CONCLUSION

The use of salivary biomarkers for stress research is
increasing based on the convenience of collection, affor-
dability and scientific merit. Instead of considering
salivary biomarkers as an alternative to blood, it is
increasingly being recognized as a legitimate source for
analyzing the stress response in humans.

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