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Salivary Biosensors for Screening Trauma Related Psychopathology

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Summary

Following facial trauma, a distinct subset of patients will go on to develop mental health problems including recalcitrant psychopathology. Early identification of maladaptive stress reactions provides opportunities for initiating preemptive mental health interventions and hinges on the surgeon's ability to differentiate between transient distress and precursors of recalcitrant psychiatric sequelae. The comprehensive care of injured patients will benefit greatly from objective adjuncts and decision-making tools to complement the clinical evaluation. This chapter addresses meeting the need for practical, standardized and reliable screening strategies through the use of promising developments in the use of stress response biomarkers and biosensing technology. The systematic interrogation of differentially expressed stress response biomarkers in saliva now permit rapid, assessment of the psychopathological response to the stressor. Quantitative, point-of-use measurements of the traumatic stress response will greatly improve the nosology of post-traumatic stress disorders, and help advance the screening, diagnosis, treatment, and prevention of mental health consequences of violence and trauma.

Introduction

As manifest by the preceding articles, facial injuries can exact a sustained psychological toll in vulnerable patients. Most individuals do recover, however, a distinct subset continues to suffer from the psychological stress of the trauma exposure and develop distress and psychiatric illness or exhibit health risk behaviors, such as substance use, that may increase their risk of reinjury. Despite the adverse impact of post-trauma psychopathology on physical recovery, quality of life, as well as social and vocational functioning, most individuals who develop these mental health problems are not identified or treated early on. A growing body of research indicates that early identification of maladaptive stress reactions provides opportunities for initiating preemptive and targeted mental health interventions. Researchers such as Noy et al. (1984)¹ have suggested that mental health interventions in the early stages may prevent the crystallization of abnormal stress reactions into entrenched psychiatric sequelae. However, the clinical challenge is one of differentiating between transient distress and precursors of recalcitrant psychiatric sequelae. Existing psychological

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screening strategies tend to be time and resource intensive and often not compatible with fast-paced trauma care. Even those clinicians and health care systems interested in providing integrated care to patients with facial injury face decisions on how best to distribute limited resources in an era of growing budgetary constraints. Which patients will need additional psychosocial services, what will be the nature of these services, and when and how will they be provided? Practical and brief screening strategies that facilitate early identification and targeted interventions will go far in directing limited health care resources to those least likely to recover without extensive professional support.

Beyond these logistical and budgetary constraints, the development of collaborative trauma care practices suffer from a lack of training in eliciting information about psychosocial issues or surgeon attitudes about collecting data seemingly unrelated to the restitution of the physical injury (Chandra et al. 2008)². Unlike the swelling, bruising, and radiographic findings that signal a bone fracture, the signs and symptoms of maladaptive stress reactions and ensuing substance use disorders are more subjective and are characterized by a vast range of behaviors. These challenges were evident in our research study (Murphy et al. 2008)³ that determined that oral and maxillofacial surgery residents treating facial injury patients did not identify alcohol use in 42% or drug use in over 65% of individuals with substance use behaviors. Current screening practices are unreliable because they rely exclusively on patient self-reports and subjective interviewer assessments of psychopathology. The problem is further compounded by the burdens of interpreting the screening results. Absent objective help, clinicians have only their judgment to resolve their clinical impressions, all of which needs to be accomplished in a trauma care setting with other competing agendas and demands. Thus, it is evident that the provision of comprehensive care for patients with orofacial injuries suffers greatly from a lack of objective adjuncts and decision-aid tools to complement the clinical evaluation.

The current chapter addresses meeting the need for practical, standardized and reliable screening strategies through the use of promising developments in the use of stress response biomarkers and biosensing technology.

Psychobiology of the Traumatic Stress Response

Based on the knowledge that the stimulus of the traumatic stressor precipitates a series of compensatory mechanisms expressed as discernible physiological changes, increasing attention has focused on identifying and measuring the corresponding biological indicators (Delahanty and Nugent, 2006)⁴. Characterizing the biological correlates of normal and abnormal psychological responses to the trauma could be very useful in the identification of emerging psychopathology and in directing referrals and treatment. Broadly, the stress response to injury is characterized by a complex and counterbalancing set of hormonal responses in the two arms of our neuroendocrine system: the sympathetic nervous system (SNS) and the hypothalamic-pituitary-adrenal (HPA) axis. Immediately following a traumatic stressor, the SNS responds by triggering the adrenal glands to release epinephrine and the sympathetic nerves to squirt out the epinephrine-like chemical norepinephrine all over the body: nerves that wire the heart, salivary glands, gut and skin. The sympathetic stress response increases the heart rate, blood pressure and blood glucose levels in muscles and vital organs to help the body adapt to the increased demand. The HPA axis response to the stressor stress is slower (i.e. minutes) and marked by an increase in the release of corticotrophin releasing hormone (CRH) and other neuromodulators from the hypothalamus. CRH stimulates the anterior pituitary gland to release adrenocorticotrophin hormone (ACTH) which in turn stimulates the adrenal glands to release cortisol as well as dehydroepiandrosterone (DHEA) and its metabolite (DHEA-S). Cortisol has an important role in shutting down sympathetic activation and suppressing the HPA axis through a

negative feedback mechanism. The anti-glucocorticoid properties of DHEA-S are believed to contribute to an upregulation of HPA-axis responses as well as mitigate possible deleterious effects of high cortisol levels on the brain (Rasmusson et al., 2003)⁵. Once the perception of threat recedes, the negative feedback mechanisms help restore hormone levels to baseline. However, if the stressors are extreme or chronic, the homeostatic process may become dysregulated and provoke the altered neuroendocrine patterns associated with various psychopathological conditions (McEwen, 2002)⁶. Considerable evidence shows a link between neuroendocrine dysregulation and psychopathology, including mood and anxiety disorders (Stratakis and Chrousos, 1995⁷; Ehrling et al. 2008⁸)

Saliva as a source of stress biomarkers

The centrality of hormonal “stress mediators” to normal and maladaptive stress responses render them an attractive means of connecting the stress experience with the individual’s psychobiological response to trauma. However, the intrusiveness and logistical limitations inherent to the pervasive use of blood and urine as sources of peripheral biomarkers have led to the growing interest in the use of saliva as an alternate (see review by Wong, 2006)⁹. A virtual mirror of the body, saliva can reflect practically the entire spectrum of normal and disease states including tissue levels of natural substances as well as hormonal and immunological status (Mandel, 1993)¹⁰. The three major salivary glands (parotid, submandibular and sublingual) and numerous minor glands produce ample amounts (500 to 1500 ml) of saliva daily (Navazesh, 2003)¹¹. For patients, supplying a saliva sample evokes less anxiety than providing a blood sample, and less embarrassment than producing a urine specimen. For clinicians and lab technicians, saliva poses much less risk of exposure to pathogens such as HIV or hepatitis than blood tests. Unless visibly contaminated with blood, human saliva is not considered a class II biohazard (US Centers for Disease Control), affording researchers and institutions both administrative and safety benefits. Unlike the phlebotomy skills required for blood collection, saliva samples are easily procured. Multiple sampling over the day or over many days can be readily completed in the field or at home, thus increasing the feasibility of doing longitudinal studies (Holm-Hansen et al., 2004)¹². Saliva requires minimal manipulation because it does not clot, and raises fewer ethical concerns than more invasive methods. Furthermore, saliva samples can be obtained without difficulty from children (Shimada et al. 1995)¹³ and individuals with physical or mental handicaps (Samuels et al. 1997)¹⁴. The non-invasive collection of saliva is particularly advantageous when subjects require regular monitoring with repeated sampling. In summary, saliva is an ideal biofluid for biomarker discovery and profiling of mental health disorders and a promising basis for inexpensive, non-invasive, and easy-to-use diagnostic technology.

Putative Salivary Stress Biomarkers

The search for salivary indices of the individual stress response has involved various components of the human salivary proteome including cortisol, DHEA-S, testosterone, catecholamines, immunoglobulin A (IgA) and chromogranin-A. Much of the attention of stress researchers has focused on salivary cortisol as an expression of HPA axis activation (Yehuda, 2005¹⁵, 2006¹⁶; Breslau, 2006¹⁷). Assessment of cortisol levels are compounded by a natural diurnal variation - high in the morning and low at night. Cortisol is thought to enter saliva by passive diffusion and correlates closely with the free physiologically active serum cortisol fraction (Kirchbaum et al., 1994)¹⁸. Unlike cortisol, conjugated steroid DHEA-S enters saliva via ultrafiltration through the tight junctions between acinar cells and has a serum-saliva correlation of 0.86 (Salimetrics). Salivary testosterone levels are significantly correlated ($r = 0.71$) with serum testosterone levels in males and stressors tend to decrease the testosterone levels (Elman & Breier 1997)¹⁹. This inverse relationship

between stress and testosterone levels was substantiated independently by Opstad (1992)²⁰ and Morgan et al., (2000)²¹ in soldiers participating in military endurance training courses.

Because salivary catecholamines are poorly correlated with plasma concentrations, they are not considered as useful index of general sympathetic tone (Chiappin et al., 2007)²². An attractive alternate biomarker of adrenergic stimulation is salivary alpha amylase (sAA). Mostly synthesized by the serous acinar cells of the parotid gland, amylase is one of the principal salivary proteins and accounts for 10 – 20% of the total salivary gland-produced protein content. Salivary glands are innervated by both sympathetic and parasympathetic nerves and secrete sAA in response to neurotransmitter stimulation (Garrett, 1999²³). Several investigators (Kyriacou et al., 1998²⁴; Rohleder et al., 2004²⁵) have demonstrated that SAA concentrations are closely associated with plasma catecholamine levels, particularly norepinephrine (NE), and are highly correlated with NE changes in response to stress. Numerous studies (Gillman, 1979²⁶; Nater et al., 2005²⁷, 2006a²⁸, 2006b²⁹) have shown that sAA levels increase under a variety of physical and psychological stressors. Salivary alpha amylase levels were found to respond to psychological stress (Bosch et al., 1996³⁰, 1998³¹, 2003³²; Skosnik et al., 2000³³) or relaxation interventions (Morse et al., 1981³⁴; 1983³⁵). Salivary chromogranin A, co-released with serum catecholamines, has also been investigated as an alternate indicator of the psychosomatic stress response (Nakane, 1998)³⁶. Yet other researchers have attempted to link salivary IgA (SIgA) levels with the psychological reaction. Tsujita and Morimoto (1999)³⁷ showed that SIgA levels vary temporally with the stress response - high in the acute stress phase and decreased in the presence of chronic stress. Whether these salivary proteins occur as a consequence of the psychological distress or are etiopathological to the stress reactions, it is increasingly evident that they are part of the biological substrate of psychological disorders. Linking these putative biomarkers to various trauma-related psychopathology (biomarker qualification) through prospective clinical studies could greatly facilitate integrated care of injured patients.

“Point of use” measurement of salivary stress indicators

Salivary biomarkers could play an essential role in trauma care by providing useful information about the patient’s psychological condition, supporting treatment decision making and also help understand the mechanisms and evolution of adverse mental health sequelae. The promise of salivary stress biomarkers notwithstanding, their clinical utility has been restricted by the lack of appropriate technology platforms that allow near “real-time” detection and quantification of these biological response indicators. Integrating early identification, risk stratification and facilitated referrals of injured patients with maladaptive stress reactions will require that the care providers have timely access to salivary stress biomarker data. Typically, biological samples collected by patients are processed in centralized laboratories which results in extended reporting times and is fraught with several potential quality failure points. For example, the total process to deliver a salivary test result involves the multiple steps of sample acquisition, labeling, freezing, transportation, processing in the laboratory (centrifugation of the sample, sorting, aliquotting, loading into analyzer), analysis and results reporting. The costs associated with expensive analytical equipment and testing supplies, sample acquisition and transport supplies, as well as all the labor costs incurred across the total process can be significant impediments to routine use of salivary biomarkers. Finally, the relative stability of a particular biomarker can dramatically impact the measured levels over the collection-measurement-reporting cycle.

In contrast, the utility of point-of-care (POC) testing of saliva resides in the immediacy of the results reporting. The ability to readily measure salivary stress biomarkers at the site of patient care increases the likelihood that the data will be utilized by the care provider to

inform clinical decision making and provide appropriate referrals. Moreover, the operating advantages of such point-of-care instrumentation will greatly facilitate future validation studies of putative salivary stress biomarkers. Developments in biosensing technology by our research group now allow the fabrication of versatile biosensing platforms (Yamaguchi et al. 2003)³⁸ for low-cost, point-of-use devices that can measure and profile putative salivary correlates of the stress response (Yamaguchi et al. 2004³⁹, 2006a⁴⁰, 2006b⁴¹, 2007⁴², 2007⁴³). Cheaper, smaller, faster, and smarter POC devices have increased the use of POC approaches by making it cost-effective for many diseases. Embedded system software process measurable biochemical signals into simple digital feedback displays readily accessible to even nonspecialists.

Salivary Biosensors as Screening Tools

In its very essence, a salivary biosensor is small, self-contained device which utilizes biological reactions for detecting and measuring a particular substance (analyte) of interest. The biosensor consists of a biological recognition element (interacting with the target analyte) in intimate contact with a transducer that translates the biorecognition event into a useful electrical signal. The common used transducers include optical, electrochemical or mass-sensitive elements and generate light, current or frequency signals, respectively. Depending on the nature of the recognition event, the biosensing platform may be either bioaffinity or biocatalytic. Bioaffinity devices rely on the selective binding of the target analyte to a surface-confined capture element (e.g. antibody, oligonucleotide). Biocatalytic devices, in contrast, utilize an immobilized enzyme for recognizing the target substrate. When exposed to a saliva sample, the interaction of the analyte with the bioreceptor produces a detectable effect that is measured by the transducer and converted into a measurable output such as an electrical signal. The strength of electrical signal is proportional to the level of the single analyte or group of analytes and the result is provided on an optical display. Figure 1 illustrates the conceptual principle of the biosensing process. Depending on the biomarker of interest, the biosensing platform may utilize antigen/antibody binding, nucleic acid interactions or enzymatic interactions to recognize the analyte. The more common forms of transducers tend to utilize optical detection (luminescence, absorption, surface plasmon resonance, etc) or electrochemical detection methods.

Table 1 and Figure 2 summarize the underlying principles of sensor devices used for the detection of salivary biomarkers. The complexities and specificities of the biosensing process increase in the following order; enzymatic methods; antigen-antibody methods; and hybridization methods, while the cost of the tests increase correspondingly. The increasing availability of enzyme-linked immunosorbent assay (ELISA) kits for measuring a range of salivary analytes (e.g., Salimetrics, LLC PA, USA) has led to resurgence in the interest in salivary stress biomarkers. Gold colloid based methods such as Localized Surface Plasmon Resonance (LSPR, Figure 2a) allow for low-cost qualitative tests manifesting the exhibiting the color reactions of ELISA as a visible line on a biochip. Similarly, enzyme sensors (Figure 2b), immuno-sensors and surface plasmon resonance (SPR, Figure 2c) display the color reactions of the ELISA process by way of electro-chemical or optical phenomena, and thus, allow measurements that are both high-sensitive and continuous. The DNA chip (DNA microarray) is a relatively new tool used to identify mutations in genes. The DNA chip, which consists of a small glass plate encased in plastic, is manufactured in a manner similar to a computer microchip (Figure 2d). The surface of each chip can contain thousands of short, synthetic, single-stranded DNA sequences. When exposed to a biofluid, competitive hybridization occurs between the immobilized synthetic cDNA/cRNA strands and mRNA/DNA in the sample. Radioactive or fluorescence tagging makes automatic detection

possible. Because chip technology is still relatively new, it is presently only used a research tool.

To realize the diagnostic promise of salivary biomarkers for identifying patients at risk for psychopathology after traumatic exposure, we have been developing hand-held, point-of-care biosensors which can be used in a variety of acute care settings. Our design challenge was to develop a low-cost biosensor whose operation is simple and robust enough to deliver laboratory accuracy and reliability in locations far less well controlled than the laboratory. Because the early adrenergic response has been implicated in the development of traumatic psychopathology, we have focused initially on the measurement of salivary alpha-amylase which reflects sympathetic nervous system (SNS) activity. Our biosensor system design uses an inexpensive ($\approx$ \$1) disposable, plastic saliva collection strip and a hand-held reader (Figure 3).

Briefly, the saliva collector at the tip of the test strip is placed under the tongue, allowed to saturate with saliva ($\approx$ 10 seconds) and inserted into the reader. This act activates the reader and initiates a transfer of the collected saliva onto the biosensing platform where the transferred sAA metabolizes a chromogenic substrate to yield a colored product. The embedded microprocessor (MPU) notes the activation of the reader as the initiation of the reaction time ($t = 0$ s). At $t = 10$ seconds, an alarm indicates the end of saliva transfer and the collector/strip is removed from the reader. At $t = 20$ seconds, the reflectance of the product of the enzyme reaction is measured photometrically and the SAA levels reported on the display along with a date and time stamp. Normalizing equations for temperature ($R^2 = 0.99$) and pH ($R^2 = 0.96$) inputted into the biosensors MPU minimize the impact of variations in ambient temperature and salivary pH. An embedded miniature thermosensor measures the ambient temperature at the time of saliva collection and the temperature adjustment equation within the biosensor MPU transforms the measured values into SAA activity at 37°C . The single use plastic strips, similar to the paper strips for glucose monitoring of diabetics, is based on dry reagents and allows considerable simplification of the analytical system and freedom from complex maintenance, calibration, and quality control procedures.

Performance Characteristics of Salivary Biosensor Prototype

A fundamental quality of any salivary biosensor is its ability to provide reliable analytical results in a variety of field conditions. Thus, verification of the performance characteristics (analytical validation) of a biosensor should include assessment of its accuracy (i.e., how closely do the biosensor readings compare to a gold standard method) and precision (i.e., how reproducible are the biosensor measurements). We verified the accuracy of the SAA biosensor prototype by establishing the correspondence between the portable SAA biosensor and the “gold standard” – a conventional, laboratory-based Olympus 400AU clinical chemistry analyzer. Briefly, 20 normal, healthy subjects provided saliva samples by passive drool. The SAA levels in the individual samples were determined using 5 biosensor prototypes compared to the Olympus analyzer. Figure 4 summarizes the consistent readings provided by the individual biosensors and the strong positive correlations ($r = 0.989$) between the SAA biosensor readings and the Olympus analyzer. Fitting a linear regression model predicting biosensor readings from the “gold standard” produced a slope estimate = 1.09 and an $R^2 = 0.98$ (Figure 5). An intra-class correlation coefficient (ICC) of 0.97 indicated that less than 3% of any measurement variability could be attributable to biosensor. Simply stated, if a number of the SAA biosensors were deployed in field studies, the measurement variability across the biosensors (i.e. the noise) would be minimal when compared to the natural variability in SAA levels in the study population.

Reproducibility of SAA biosensor measurements was evaluated by repeating the analysis of 5 saliva samples after 6 weeks (Figure 6). There was no apparent “drift” in the data as the measurements across the testing points did not appear to differ in terms of their bias, mean, or variation. Thus, based on these biosensor validation studies, it appears the SAA biosensor prototypes are reliable and possess the precision, accuracy and reproducibility required for routine point-of-care use in trauma settings.

Clinical Implications

The psychological burden of traumatic injury in general, and orofacial injury in particular, provide a compelling rationale for an integrated care approach that addresses all aspects of the recovery process. The use of putative biological correlates to rapidly identify and differentiate between normal or pathogenic psychological processes has the potential to fundamentally change the way care is delivered to trauma patients. Such an approach would be more reliable, practical and informative about the pathogenesis and trajectory of maladaptive stress reactions than the existing subjective way of assessing psychopathologic parameters. Surgeons, alerted to evolving morbidity through point-of-care testing of salivary stress biomarkers, would be positioned to make timely treatment decisions and referrals for targeted interventions. A biomarker-based strategy would also be very useful for tracking disease progression and monitoring response to any treatment. The clinical utility of stress biosensors is predicated on the notion that select salivary biomarkers can serve as indicators of normal or pathogenic processes. An improved awareness of the psychological impact of facial injury could drive the discovery and validation of additional biomarkers for screening psychiatric disorders. Research studies correlating the dynamic alterations of the salivary proteome with the psychological changes related to mental health sequelae will support integrative models for understanding risk and resilience to traumatic psychopathology. The search for peripheral markers of stress to complement clinical evaluation is not unlike biomarker discovery in other specialties such as cardiac transplantation, where overexpression of myocardial proteins such as troponin is used to predict the onset of rejection several weeks prior to conventional histological diagnosis. Quantitative measurements that provide information about biological processes will greatly improve the nosology of post-traumatic stress disorders, and help advance the screening, diagnosis, treatment, and prevention of mental health consequences of violence and trauma.

Future Perspectives

The application of a salivary stress biomarker strategy to trauma care hinges on the development of devices and technologies that allow systematic measurement and reporting of target biomarkers. The rapidly evolving disciplines of proteomics, bioengineering, analytic chemistry and computational biology are producing a variety of new tools and biosensing platforms that are well-equipped to monitor the presence or absence of an increasing array of biomarkers. Indeed, some have already been deployed into devices that are already employed in clinical settings for the diagnosis and/or prognosis of certain diseases (Song et al., 200644). Our preliminary biosensor development suggests that the measurement and interpretation of salivary proteome levels by hand-held biosensors is a practical, reproducible, less invasive, inexpensive and point-of-use alternate to the invasive and labor-intensive blood or urine profiling approaches currently utilized by traumatic stress researchers. One of the most rapidly advancing of these fronts is the area of multianalyte biosensing which acknowledges the substantial heterogeneity among psychiatric sequelae and the corresponding inadequacy of a “single biomarker” approach for measuring and tracking severe stress reactions. Future developments in biosensing technologies that allow systematic interrogation of differentially expressed salivary proteins will permit assessment of each psychopathological response by cross-referencing it to at least three neurohormonal

markers linked to mental health risk and resilience (Charney, 2004)⁴⁵. We anticipate that prospective clinical studies will lead to the development of libraries of executable algorithms and treatment decision trees. Once inputted into the biosensor microprocessor, these algorithms could analyze patterns in salivary stress biomarkers to generate simple feedback displays readily accessible to even non-specialist personnel. Such “smart systems” will bridge the gaps created by the relative lack of mental health specialist in trauma care settings because the processed output will not require specialized training or knowledge to interpret or implement. Enabled by this technology, surgeons and other health care providers will be able to conduct timely restorative interventions or refer “at-risk” individuals for specialized mental health care to protect against untoward psychiatric reactions. The maturation of the salivary biosensors into commercial devices will happen as the functionality, performance and production costs improve. In the long term, we expect that continuing advances and a growing convergence of proteomic and genomic profiling, biosensing technology and bioinformatics will create a paradigm shift in the understanding, diagnosis and management of traumatic stress and psychopathology.

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Figure 1.
Components of a typical biosensor.

Figure 2.

Illustration of various biosensing approaches for the detection of salivary biomarkers. (a) Local Surface Plasmon Resonance; (b) Enzyme sensor; (c) Surface Plasmon Resonance; (d) DNA chip.

Figure 3. Saliva collection strip and prototype biosensor for point-of-use measurement of salivary alpha-amylase (sAA).

Figure 4.
Concordance between sAA biosensors and Olympus analyzer.

Figure 5.
Linear regression fit to mean biosensor values.

Figure 6. Reproducibility of sAA biosensor verified by repeat measurements of 5 saliva samples. Negligible variation from the “true value” (horizontal lines representing the Olympus AU400 readings) indicate minimal measurement drift.

Table 1

Principles of Biosensor Detection

Biological Recognition Element	Biomarker	Sensor device
Enzymatic	Enzyme Substrate	Test paper (Dry chemistry) Enzyme sensor (Electrochemical sensor)
Immunoassay	Antigen and Antibody	ELISA
	Hormone	Gold colloid method (LSPR)
	Neurotransmitter	Electrophoresis
	Enzyme	Immuno-sensor (Electrochemical sensor)
	Xenobiotics	SPR
Hybridization	mRNA, DNA	DNA chip

ELISA: Enzyme-linked Immunosorbent Assay, LSPR: Local Surface Plasmon Resonance, SPR: Surface Plasmon Resonance