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The Role of Salivary Neuropeptides in Pediatrics: Potential Biomarkers for Integrated Therapies

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Abstract

Introduction—Objective measures of symptom response to integrated complementary approaches in pediatrics are evolving. The purpose of this study was to document the concentration range of salivary neuropeptides in healthy controls and in children with cancer, to explore correlations between serum and salivary measurements for Calcitonin Gene-Related Peptide (CGRP) and Vasoactive Intestinal Polypeptide (VIP), and to determine whether there is a change in these salivary neuropeptide levels in response to integrated mind-body therapies.

Methods—A non-randomized pragmatic study with three phases: Phase 1- Healthy Control Saliva-10 healthy controls provided saliva samples; Phase 2- Cancer Diagnosis Serum-Saliva- 16 mixed-type cancer patients provided blood and saliva samples; Phase 3- Acute Lymphocytic Leukemia (ALL) Saliva Intervention- 12 patients with ALL provided pre- and post-complementary intervention saliva samples.

Interventions—20-minutes of structured touch or scripted relaxation breathing were administered to patients in Phase 3; Phase 1 and 2 patients did not receive this intervention.

Outcome Measures—cortisol, CGRP, VIP, State/Trait Anxiety Scale, visual analogue scale, vital signs.

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Results—Salivary CGRP and VIP were similar for children in Phases 1 and 2. There was a correlation between serum and salivary VIP in the mixed cancer group, though not between serum and salivary CGRP. In Phase 3 children, following a complementary intervention, salivary CGRP, heart rate, and systolic blood pressure decreased.

Discussion/Conclusions—These data provide evidence of a decrease in sympathetic output after integrative/complementary therapy intervention in children with cancer. The study underscores the potential role of salivary neuropeptides as non-invasive biomarkers for integrated therapies in pediatrics.

Keywords

Salivary neuropeptide; integrated therapy; complementary therapy; biomarker; autonomic nervous system

Introduction

Despite the growing popularity of complementary and alternative medicine (CAM), or “integrated”, approaches in pediatrics, deciding upon an appropriate, effective complementary therapy for an individual child with a specific diagnosis remains primarily empirical and practitioner- dependent.[1, 2] Examining the complex mechanisms involved in response to these interventions and identifying reliable, objective, and non-invasive biomarkers that correlate with relief of common symptoms, such as pain and anxiety, are important next steps toward evidence-based therapy in the pediatric population.

The role of the autonomic nervous system (ANS) in response to stressors, such as pain and anxiety, has been well-documented. The release of neurotransmitters, noradrenaline from sympathetic and acetylcholine from parasympathetic synaptic terminals, influences not only visceral functions, including heart rate, blood pressure, and release of cortisol, but also release of neuropeptides (NPs). Direct measurement of noradrenaline and acetylcholine released from vesicles at neuro-effector junctions can be done experimentally. Clinical measurement of plasma neurotransmitters requires venipuncture and special handling. However, NPs, which function as mediators of both the sympathetic and parasympathetic branches of the ANS, may be measured in a variety of body fluids, including blood [3], sweat, tears, saliva, and semen. [4]

The NPs, Calcitonin Gene-Related Peptide (CGRP) and Vasoactive Intestinal Polypeptide (VIP), belong to the group of non-adrenergic, non-cholinergic transmitters existing within nerves in the salivary glands. [5,6] In salivary glands, VIP co-localizes with choline acetyltransferase and has been used as a peptide marker for post-ganglionic parasympathetic fibers [7]. In the neurovascular model of migraine, trigeminal activation leads to activation of the parasympathetic pathway, the so-called trigeminal-autonomic reflex arc, that results in release of neurotransmitters. Thus, a reflex connection exists between neurons in the pons (superior salivatory nucleus), which results in a cranial parasympathetic outflow that is mediated through the pterygopalatine, otic, and carotid ganglia. This reflex is present in normal persons and appears to be active during migraine and possibly allergic rhinosinusitis. [8] CGRP has been correlated with sympathetic (trigeminal) activity, selectively stimulates

noradrenergic sympathetic outflow, and plays a role in nociception, or the ability of the body to sense potential harm. [9] Santavirta examined the activity level of the ANS using salivary neuropeptides (sNPs) in adults with Sjogren's syndrome and found a significant correlation of increased concentrations of VIP with higher stress measures. [10] Bellamy et al. showed that elevated salivary CGRP and VIP correlated with trigeminal and parasympathetic nerve activity in adults with migraine headaches between and during attacks, as well as with response and return to baseline with specific pharmacologic treatments. [11] To our knowledge, no comparable studies have been undertaken in children.

Salivary cortisol is an acceptable non-invasive measure of adrenal cortical function and indirectly, the sympathetic stress response. [12-14] However, limitations of salivary cortisol, such as performance variability among different tests and conflicting evidence regarding interpretation of values in the face of clinical use of steroids, underscore the importance of the identification of alternative methods for non-invasive measurement. sNPs are ideal candidates to perform this role.

Although the beneficial effects of massage therapy in both children and adults involve mediation through the parasympathetic nervous system, this therapy is not always practical or accessible. The "M" technique, is a method of gentle touch involving a series of stroking movements performed in a set sequence. [15] It has been shown to be reproducible in research protocols involving both neonates and adults, when provided by a certified practitioner. The use of the "M" technique in premature neonates resulted in decreased stress indicators, including heart rate, respiration, oxygenation, and behavioral state.[16, 17] A pilot study of "M" technique following neonatal circumcision showed decrease in neonatal pain scale and improved oxygenation. Adult patients had changes in heart rate variability consistent with an increase in parasympathetic response. [18]

Various combinations of relaxation breathing and progressive muscle relaxation have shown positive impact on outcomes of perceived health status, decreased pain, and improved functioning in children with asthma and rheumatologic disorders.[19-21] Relaxation training is often a part of behavioral protocols for pediatric patients as it impacts pain and/or anxiety. Studies in adults with cardiac diagnoses [22] and other diseases treated with various alternative breathing techniques [23] suggest an increase in parasympathetic activity. Sakakibara's study with adults found that a muscle relaxation exercise increased parasympathetic tone [24] and in asthma patients, progressive muscle relaxation produced significant increases in vagal parasympathetic tone. [25]

The use of sNPs as biomarkers for sympathetic/parasympathetic response to a therapeutic intervention, particularly in pediatrics, is an emerging concept. [9-11] Our proposed investigation into the non-invasive measurement of these ANS co-transmitters in the saliva of pediatric patients is a novel application of an existing methodology to a population not previously studied. It is currently unknown: 1) what is the range of salivary CGRP and VIP measurements in healthy children or children with cancer?; 2) is there a correlation between serum and salivary NPs (CGRP and VIP) in the pediatric age group?; and 3) is there a change in these sNPs in response to complementary mind-body therapies? We conducted a

non-randomized pragmatic study in order to collect preliminary data in anticipation of a larger randomized clinical trial.

Materials and Methods

Recruitment of patient subjects, as well as appropriate consent/assent, took place with the assistance of Clinical Trials Office staff in the Pediatric Oncology Clinic at Primary Children's Hospital. All research protocols were approved by the Clinical Cancer Investigations Committee based at the Huntsman Cancer Institute, the University of Utah, the privacy board of Primary Children's Hospital, and the Institutional Review Board of the University of Utah. All patients and healthy controls were recruited from November 2011-December 2012 and were between 9 and 18 years of age. Children of both genders were included.

Study Protocols

Collection of Serum for Neuropeptides

Collection of Blood: Blood was obtained during sterile port access for other routine blood work at the time of the oncology visit, spun in the hospital lab, and serum removed and frozen (-20°C) until ready for dry ice shipment to the laboratory at Missouri State University.

Collection and Analysis of Salivary Proteins and Neuropeptides

Preparation of Tubes: Saliva collection tubes were pre-labeled with a patient code and sample number by the laboratory at Missouri State University. They were placed in a freezer (-20°C) in their transport case (1 case per patient) prior to collection to ensure that tubes were cold and ice packs were frozen. After collection, tubes were immediately returned to the freezer.

Preparation of the Mouth: Patients were advised to avoid consumption of food and drink for two hours before collection of saliva. Patients expectorated their mouth of saliva and rinsed with water immediately prior to sample collection.

Collection of Saliva: The saliva was collected at a consistent time of day (between 10 a.m. and 2 p.m.). Saliva collection and analysis was performed as previously described with the following modifications.[11] The transport case assigned to the patient was opened and the patient given a piece of gum from the case and instructed to gently chew a piece of citric flavored gum for five minutes. During the first two minutes, saliva was discarded to prevent the mixing of unstimulated and stimulated saliva. While continuing to gently chew the gum, this stimulated saliva was expectorated into a chilled 15 mL conical tube until 5 mL of saliva was collected, and the tube immediately placed back in the pack and frozen (-20°C) until ready for shipment on dry ice.

Processing of the Serum and Saliva Samples—Serum and saliva samples were analyzed following manufacturer's instructions using commercially available kits for detection of human CGRP and VIP (RIA; Bachem, Torrance, CA, USA) and human cortisol

(ELISA; Cayman Chemical, Ann Arbor, MI). Values were normalized to total volume of sample collected and total amount of protein as determined by the Bradford method. [26]

Mann-Whitney U-tests were performed for comparisons of CGRP, VIP, and cortisol levels between groups, and time points using IBM SPSS V20. Statistical significance was considered at $p < 0.05$.

Implementation Protocols

We conducted a three phase study. All patients received a \$25. gift card at the conclusion of their study participation.

Phase 1

Healthy Control Saliva Arm: We recruited 10 healthy control children from community youth groups. The enrolled children provided 2 saliva samples each, taken at the same time of day on 2 separate days for measurement of cortisol, CGRP and VIP. The results for each patient were averaged.

Phase 2

Cancer Diagnosis Serum-Saliva Arm: Sixteen patients with various diagnoses of pediatric cancer, both blood and solid tumor, consented to simultaneous serum measurements of CGRP and VIP and saliva measurements of cortisol, CGRP, and VIP at the time of a routine visit for chemotherapy and/or monitoring of their disease. All samples were obtained prior to any procedures or treatments that were performed on the day of collection.

Phase 3

Acute Lymphocytic Leukemia (ALL) Saliva Intervention Arm: Twelve patients with ALL in the maintenance phase of chemotherapy were enrolled. Samples for pre-intervention salivary cortisol, CGRP, and VIP were first collected. The patients then underwent their choice of complementary intervention and State-Trait Anxiety Inventory (STAI) [27, 28] and visual analogue scale for pain (VAS) for pain (as described below), as well as vital signs. Salivary cortisol, CGRP, and VIP were obtained immediately following the intervention. The time between pre- and post-intervention sample collection averaged 45-minutes and was not statistically different for any of the patients.

Intervention: Ten patients were exposed to a 20-minute session of “M” technique for hand and feet. Two patients declined touch and requested a standardized relaxation breathing script instead. Once patients were enrolled, initial vital signs were obtained. Measurements were taken by trained technicians or registered nurses; blood pressures were obtained by Dynamap with appropriate size cuff. The baseline STAI, which is a validated measure examining both constitutional and situational or anticipatory components of anxiety, was administered along with a VAS as anxiety may overlap with fear as a response to a previous painful experience. There is a systemic release of corticosteroids that accompanies stress with the resultant autonomic response seen as increased heart rate, increased arousal, and anxiety. [29] Measurement of anxiety that takes into account the personality of a child when scoring may be accomplished by the STAI. As there are age-related differences in

perceptions of anxiety, the STAI-C was used for ages 9-12 and STAI for above age 12. The STAI is written at a sixth-grade reading level and is easy to read; the STAI-C for younger children may also be administered verbally. The VAS, or Graphic Rating Scale, uses a visual format with a metaphor of distance and relative position to represent importance. For the purposes of this study, the chosen test displayed a scale as a blank

line with an extreme facial (“happy” versus “sad”) depiction placed on either end of the line. The patient was instructed to place an “X” somewhere between the two marks to indicate: “How much pain are you currently feeling?” Analysis was made by making simple divisions across line width in millimeters.

Results

Descriptive statistics regarding the three study arm phases appear in Table 1.

Phase 1

Salivary cortisol and sNPs were measured on two consecutive days, at the same time each day, in a group of ten healthy control children to determine reproducibility. Measurements for salivary cortisol, salivary CGRP, and salivary VIP were similar on both collection days (data not shown). Salivary cortisol and sNPs in healthy controls are shown in Table 2a.

Phase 2

Salivary cortisol and sNP results for patients in the mixed cancer diagnosis group are shown in Table 2b. Of the 16 patients, there were six insufficient samples for cortisol and two samples insufficient for CGRP and VIP. There were no statistical differences between the healthy control group and this group for the parameters examined.

Serum and salivary CGRP are compared in Figure 1a. Statistical significance was not reached ($p=0.096$). However, there was a close correlation between serum and salivary VIP as seen in Figure 1b ($r^2= 0.792$, $p=0.002$).

Phase 3

In this phase, we sought to determine whether sNPs correlated with the ANS response in children with ALL following a brief integrated therapy intervention. We compared pre- and post-intervention of salivary cortisol, salivary CGRP, and salivary VIP (Table 3a). Of these three comparisons, only salivary CGRP decreased significantly post intervention ($p = 0.0291$) while there was a trend for salivary VIP to decrease post intervention ($p = 0.0603$). Comparisons of pre/post intervention measurements of vital signs, pain, and overall state (anxiety level relating to a specific event, e.g. being in the oncology clinic) in this group are shown in Table 3b. Baseline heart rate decreased from 84.4 bpm pre intervention to 75.1 bpm post- intervention ($p = 0.0142$). Interestingly, systolic blood pressure also decreased from 116.4 mm Hg pre to 108.2 mm Hg post ($p = 0.0157$), while diastolic blood pressure did not change significantly. Patients' State anxiety measurements also decreased after therapy from 32.63 to 28.00 ($p = 0.0100$) in the eight patients who had this parameter

measured both pre- and post-intervention. Pain did not change significantly post-intervention.

Discussion

Many integrated therapies in pediatrics have shown encouraging findings in case reports and uncontrolled studies. [30-33]. Careful testing of unproven but potentially therapeutic interventions in ways that permit definitive conclusions regarding efficacy is now a research priority. Our project addresses some gaps in knowledge of the clinical application of measurement of sNPs in pediatrics. We demonstrated the feasibility to collect, process, and measure saliva for sNPs in both healthy and ill children, using a carefully implemented protocol. The values that were obtained for CGRP and VIP in children are similar to those reported in saliva from adult rhinosinusitis and migraine patients. [34, 35] We compared salivary cortisol and sNPs in healthy controls versus children with cancer and found no statistical differences. Clearly, we may have missed a significant difference due to a small sample size (Type II error). In this study, we did find strong correlation between salivary and serum VIP in children with cancer and a trend toward correlation between salivary and serum CGRP in this age group. Importantly, there was a significant measurable change in salivary CGRP associated with changes in vital sign indicators of the ANS, namely systolic blood pressure and heart rate. The decrease in CGRP implies a decrease in sympathetic output following complementary therapy intervention. The complex interactive effect of heart rate and muscle sympathetic nerve activity on systolic blood pressure has been previously documented and is coordinated with parasympathetic nervous system input. [36] Post-intervention, we would have anticipated a decrease in parasympathetic activity (salivary VIP used as marker) via the trigeminal-autonomic reflex in response to the quieting of the nervous system. Lack of significant measurable change in VIP in our study may have been due to a variety of factors, including small sample size, brief period of the intervention, delayed appearance in the saliva, diminished parasympathetic responsiveness possibly related to higher baseline cortisol, or lack of clinical relevance.

Similar correlations of serum and salivary NPs have been found in adults but to our knowledge, no prior studies have noted this association in children with cancer or in reference to response to integrated therapies. Our data extend the understanding of pediatric integrated therapies, as the use of sNPs in pediatrics may be invaluable as a tool to measure sympathetic and parasympathetic responses to a variety of therapeutic interventions. Clearly, as the current study involved a limited number of children in a non-randomized fashion, larger randomized studies are needed. The inclusion of heart rate variability as an outcome measure would add more specific information regarding the contributions of the sympathetic versus parasympathetic nervous systems. While small sample sizes make statistically significant conclusions about efficacy difficult, our study has generated data regarding methods of collection of salivary NPs in pediatrics. We have obtained baseline salivary NP ranges in healthy children and in children with cancer, noted a correlation between serum and salivary VIP, and shown the effect of complementary intervention in decreasing salivary CGRP and VIP.

Conclusions

Our study begins to address the potential role of salivary CGRP and VIP as biomarkers for integrated (CAM) therapies in pediatrics. We demonstrate feasibility of a non-invasive method for assessing the balance of the parasympathetic/sympathetic response to integrated therapies in children, thereby increasing knowledge regarding integrated mechanisms, and examining the role of sNPs in response to oncologic pain and anxiety associated with clinic visits in children with cancer.

Our project contributes to further understanding of the “biological mechanisms by which CAM therapies exert their beneficial effect thereby advancing the science and practice of symptom management.” [37] We confirm the biological relevance, with respect to the ANS, of changes in the specific sNPs CGRP and VIP, associated with easily-performed and accepted integrated therapies and provide evidence for salivary measurement as a pertinent non-invasive outcome measure in pediatrics.

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Abbreviations

CAM	complementary and alternative medicine
ANS	autonomic nervous System
NP	neuropeptides
sNP	salivary neuropeptide
CGRP	Calcitonin Gene-Related Peptide
VIP	Vasoactive Intestinal Polypeptide
ALL	Acute Lymphocytic Leukemia
VAS	Visual Analogue Scale
STAI	State Trait Anxiety Inventory

Figure 1a.
Serum CGRP vs. Saliva CGRP in cancer patients.

Figure 1b.
Serum VIP vs. Saliva VIP in cancer patients.

Table 1

Descriptive Statistics for Study Phases

Study	Number of participants	Age Range (years)	Mean Age (years)	Males	Females
Phase 1: Healthy Controls	10	9-17	13.6	4	6
Phase 2: Mixed Cancer Diagnoses	16	9-17	13.2	9	7
Phase 3: ALL	12*	9-17	13.0	10	2

* 10 received “M” technique, 2 received relaxation breathing

Table 2a
Salivary Cortisol and Neuropeptides in Healthy Controls

Variable	Range	Mean	SD	Median
Cortisol (n=10) (pg/mL)	149.21-4235.47	1468.07	1427.05	1028.36
CGRP (n=10) (pmol/mg total protein)	4.37-475.71	118.06	154.38	43.55
VIP (n=10) (pmol/mg total protein)	3.51- 230.93	61.51	86.06	14.09

Table 2b
Salivary Cortisol and Neuropeptides in Mixed Diagnosis Cancer Patients

Variable	Range	Mean	SD	Median
Cortisol (n=10) (pg/mL)	218.61-3545.54	944.93	983.28	748.63
CGRP (n=14) (pmol/mg total protein)	5.79-319.88	70.55	102.27	33.26
VIP (n=14) (pmol/mg total protein)	6.66-279.92	60.43	89.64	25.12

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Table 3a

Pre- and Post- Intervention Salivary Neuropeptides.

Variable	N	Range	Mean	S.D.	p value
pre Saliva Cortisol	9	442.59-1609.96	779.30	411.05	0.1311
post Saliva Cortisol	9	215.83-924.52	604.61	228.58	
pre Saliva CGRP	10	6.45-183.58	71.03	64.31	0.0289
post Saliva CGRP	10	6.94-45.56	23.68	14.39	
pre Saliva VIP	10	3.07-221.20	51.88	68.21	0.0603
post Saliva VIP	10	3.38-18.39	8.14	5.28	

Table 3b

Pre- and Post- Intervention Vital Signs.

Variable	N	Mean	S.D.	Min	Max	p value
Age	12	13.00	3.13	9	17	
Pre State Anxiety	12	29.42	6.95	21	41	
Post State Anxiety	8	28.00	4.54	21	33	0.0100*
Pre Pain	11	1.68	1.52	0	5	0.2910
Post Pain	11	1.00	1.40	0	4.5	
Pre HR	12	84.42	15.76	59	115	0.0142
Post HR	12	75.08	12.38	52	101	
Pre Systolic BP	12	116.42	4.94	110	128	
Post Systolic BP	12	108.17	10.96	95	127	0.0157
Pre Diastolic BP	12	67.25	4.05	61	75	
Post Diastolic BP	12	65.92	15.56	51	109	0.7534

* p-value was calculated in the eight patients who had this parameter measured both pre- and post-intervention