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## Detection of Salivary Oxytocin Levels in Lactating Women

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### Abstract

Oxytocin is a neuropeptide with widespread influence on many physiological and social functions including: labor and birth, lactation, sexual behavior, nurturing maternal behaviors, and stress reduction. However, our understanding of oxytocin's roles has been hampered by lack of noninvasive methods for assessing oxytocin levels. The goal of the present study was to assess whether oxytocin could be detected in saliva and whether changes occurred in the pattern of oxytocin release among lactating women from before, at initiation and after breast feeding. Using a prospective repeated measures design, 11 research participants each provided 18 saliva samples during three feeding cycles (before, at initiation and after breast feeding) for two 24-hour data collection periods (Day 1 and 2). Within each day, saliva was collected at late evening, early morning, and late morning. Salivary samples were concentrated four-fold by dehydration prior to analysis and oxytocin was measured in saliva using an enzyme immunoassay (EIA). Salivary oxytocin values, when reconverted to their original levels, ranged from 6.44 – 61.05 pg/ml. Oxytocin values in saliva varied significantly as a function of the breast feeding cycle, but did not show reliable differences as a function of the time of feeding. Oxytocin was highest before feeding, followed by a decrease at initiation of feeding, and an increase at 30 minutes after feeding. The findings suggest that oxytocin release into saliva increases in anticipation of feedings. This study also supports the potential usefulness of salivary measures of oxytocin as a noninvasive index of changes in this peptide.

### Keywords

Oxytocin; salivary neuropeptides; lactation; enzyme immunoassay

### Introduction

Oxytocin, a nine amino acid neuropeptide, is produced mainly in hypothalamic nuclei and released into central and peripheral systems, and is also produced in peripheral tissues including the uterus, placenta, corpus luteum, and heart (Gimpl & Fahrenholz, 2001; Kiss & Mikkelsen, 2005). The peripheral role of oxytocin in labor and lactation is well established, although much remains to be understood regarding the behavioral and cellular mechanisms of oxytocin's functions (Blanks & Thornton, 2003; Uvnas-Moberg, Johansson, Lupoli, & Svennersten-Sjaunja, 2001). Specifically, in lactation, oxytocin released during milk ejection causes the contraction of myoepithelial cells surrounding mammary gland alveoli (Riordan, 2005; Zingg & Laporte, 2003). As a neuromodulator in lactation, oxytocin appears to facilitate behavior that aids in the physiology of lactation (Matthiesen, Ransjo-Arvidson, Nissen, & Uvnas-Moberg, 2001; Uvnas-Moberg et al., 2001). Oxytocin, released during

lactation, is also associated with reduced reactivity to stressors, as well as lower heart rate and blood pressure (Altemus, Deuster, Galliven, Carter, & Gold, 1995; Altemus et al., 2001; Redwine, Altemus, Leong, & Carter, 2001). Furthermore, functional magnetic imaging and studies examining the central expression of cFOS suggest that oxytocin affects brain structures implicated in behavioral modulation and the physiology of birth and lactation (Febo, Numan, & Ferris, 2005; Lin et al., 1998).

Research on oxytocin and behavior has been generated mainly from animal models, as there are many challenges in the measurement of oxytocin in humans. These issues include the lack of a documented relationship between central and peripheral effects of oxytocin (Amico, Challinor, & Cameron, 1990). Oxytocin does not easily cross the blood brain barrier; although there may be peripheral pathways whereby oxytocin indirectly affects behavior (Uvnas-Moberg, 1998). Early literature reports inconsistent data on the metabolism of oxytocin (Leake, Weitzman, & Fisher, 1981; Thornton, Davison, & Baylis, 1990). Studies may not have controlled for the increased metabolic clearance rate of oxytocin due to the placental increase in enzymes responsible for the degradation of oxytocin (Thornton et al., 1990). Because of oxytocin's pulsatile release, it is difficult for plasma and urine sampling in humans to capture rapidly changing patterns of oxytocin concentration (Blanks & Thornton, 2003; White-Traut, Powlesland, Gelhar, Chatterton, & Morris, 1998). Also, due to the interaction between oxytocin and stress response hormones, blood sampling via a single stick venipuncture can induce a stress response, which may then suppress the release or action of oxytocin. Urinary assays restrict the study population to one that is developmentally and psychologically capable of providing accurate sampling, thus excluding infants, fragile individuals, and certain pathological populations. Researchers have attempted to develop a salivary oxytocin assay; however a widely accepted and reliable method has not yet previously been validated due to the fact that the amount of oxytocin is below the detection of most assays (Carter et al., 2007; Horvat-Gordon, Granger, Schwartz, Nelson, & Kivlighan, 2005). Due to the potential influence of oxytocin on behavior, mental health, memory, childbearing, child development and metabolism (Blanks & Thornton, 2003; Carter, 1998; Carter et al., 2006; Francis, Young, Meaney, & Insel, 2002; Uvnas-Moberg, 1997; Uvnas-Moberg, Arn, & Magnusson, 2005), a noninvasive measure is critical to advancing our understanding of factors regulating oxytocin levels under naturalistic conditions.

Lactating women provide an ideal population to conduct studies on the detection of oxytocin via salivary assays. During the milk-ejection reflex, pulsatile waves of oxytocin are released from the posterior pituitary and cause milk to be released from alveoli into ducts that open into nipple pores (Riordan, 2005). Thus, studies in lactating women offer an opportunity to measure changes in oxytocin in a population that has elevated basal oxytocin production, and also in whom oxytocin is expected to vary systematically over a short period of time.

## **Purpose**

The primary purpose of this research was to determine whether oxytocin can be detected in saliva via an enzyme immunoassay (EIA). A second purpose was to identify whether an oxytocin pattern exists during breast feeding cycle (before, at initiation and after breast feeding).

## **Materials and Methods**

### **Design**

A prospective repeated measures design was employed. Eleven research participants provided saliva samples during three breast feeding cycles (within 30 minutes before breast

feeding, at the initiation of breast feeding, and 30 minutes after completion of breast feeding) for two 24-hour data collection periods (Day 1 or Day 2). Within each 24-hour data collection period, there were three sample collection sessions: a late evening feeding, the first morning feeding, and a late morning feeding (Time of Feeding). The three time points surrounding one feeding (the breast feeding cycle) were selected to identify the change in oxytocin levels across the feeding cycle. Three feedings per day were selected to allow for a comparison of patterns of oxytocin release into saliva over three separate feedings. Day 1 and Day 2 were selected to allow for comparison of mean oxytocin levels between the two days.

### Participants and Setting

After IRB approval was obtained, 11 women were recruited into the study using a public bulletin board flyer. Data were collected in the research participants' homes.

Women were eligible if they: (1) were currently exclusively breastfeeding their infants, (2) had infants under eight months of age, (3) did not have a past or present history of endocrine disease(s), (4) did not have a cold or fever two weeks prior to data collection, (5) did not use any over the counter cold medications 24 hours prior to the data collection, (6) did not have any gum disease, and (7) could comprehend English in speaking, listening, and writing. Women were excluded if they were less than 18 years old and fed their infants breast milk, but did not suckle the infant.

Research participants were predominately Caucasian ( $n = 9$ ), with one Mexican American participant and one Asian American participant. Average maternal age was 35 years ( $SD = 3.90$ ). Average infant age was 3.67 months ( $SD = 2.08$ ). Seven infants were male and four infants were female. Six mothers were multiparous and five were nulliparous.

### Enzyme Immunoassay Method

Oxytocin level was measured via an enzyme immunoassay (EIA) using the EIA kit from Assay Designs (Ann Arbor, Michigan). Neuropeptide cross-reactivity was reported by Assay Designs as  $< 0.001\%$ . Oxidized active oxytocin was detected at a minimum level of 4.68 pg/ml. The chemical validity of the EIA was assessed using tests of parallelism, accuracy, and precision. This assay was previously used to measure salivary oxytocin in men following a shoulder massage (Bello, 2007). Following the massage, a small increase in oxytocin was detected via saliva and plasma samples ( $p < .05$ ), with oxytocin levels detected at 1 – 2 pg/ml in saliva and 150–250 pg/ml in plasma. To assess parallelism of a dilution series of saliva to the standard curve, a homogenous pool of saliva samples was made, concentrated and diluted. Precision of the assay was assessed by the variability between high and low controls (inter-assay CV) and within unknown standards (intra-assay CV). Prior to assay, saliva samples were concentrated fourfold (based on the parallelism measurements). Preliminary results indicated that oxytocin concentrations in saliva samples were usually below the lowest standard (3.9 pg/ml). To allow measurement of salivary oxytocin using a reliable portion of the standard curve, samples were concentrated before assay. Using a Lypholizer, 1 ml of supernatant from each sample was dried at 4 °C and then reconstituted in 250  $\mu$ l of assay buffer, resulting in a sample with a salivary concentration 4 times higher than the original, allowing samples within the range of sensitivity for this assay. The salivary oxytocin assay was based on prior research with voles (Kramer, Cushing, Carter, Wu, & Ottinger, 2004) and the specificity of the antibody used in the Assay Designs was determined through HPLC (high pressure liquid chromatography) prior to immunoassay using previously described methods (Carter et al., 2007).

## Procedures

After informed consent was obtained, participants were provided with the data collection materials and instructions for in-home data collection. Materials included cryovials, drinking straws, sugar and mint (noncitric) based chewing gum, leak proof plastic bags, and the data collection log.

Participants collected a total of 18 saliva samples, over two 24-hour data collection periods (Day 1 and Day 2). For each 24-hour period there were three sample collection sessions (Time of Feeding), which were a late evening feeding (the last breastfeeding before the mother put the baby to bed for the night), followed by the first morning feeding (the first breastfeeding of the day), and ended with a late morning feeding. For each sample collection session three saliva samples were collected for the breast feeding cycle. The first was within the 30 minutes before the feeding, the second at the initiation of the feeding, and the third 30 minutes after completion of the feeding. The range of days between data collection periods (Day 1 and Day 2) was 1 to 5 days (mean 2.18 days). At thirty minutes prior to each feeding, the participant rinsed out her mouth to remove food particles and drank a glass of water. Participants refrained from eating or drinking during the sample collection session. Participants collected at least 4 ml of saliva by expectorating down a straw into a test tube. If a sufficient sample was not produced within two minutes, the mother chewed on a piece of chewing gum for up to three minutes, and expectorated again. The samples were placed in leak proof plastic bags and immediately stored in their refrigerators. The participants recorded the date, time, sample number, use of chewing gum, and other comments for each sample collection session. A research team member retrieved the samples from the participants' home at the completion of the 24-hour data collection period. Upon completion of the total sample collection, participants were paid \$25 for their participation. The saliva samples were transported on ice and immediately spun in a  $-4^{\circ}\text{C}$  centrifuge upon arrival to the lab. Then each sample was stored in cryovials at  $-80^{\circ}\text{C}$  until assayed.

## Statistical analysis

Descriptive statistics were conducted on the maternal and infant demographic data. A repeated measures ANOVA was conducted to examine if there was a pattern of change in oxytocin levels during a breast feeding cycle; and if the pattern of change in oxytocin differed by the time of feeding. Time of feeding and breast feeding cycle were considered as separate variables in the analysis. Significant  $F$  ratios were followed up with post-hoc paired  $t$  tests (two-tailed) with alpha set at 0.017 using a Bonferroni adjustment (0.05/3). We conducted all of the statistical analyses using SPSS version 13.

## Results

### Samples

A total of 198 samples were collected. Inadequate saliva samples contributed toward missing data for only two aliquots, thus the final sample was 196. Mothers recorded the time of feeding. The time of feeding ranged 3 hours (from 19:17 hours to 22:35 hours) for the late evening feeding, 5 hours (02:07 hours to 07:50 hours) for the early morning feeding and 4 hours (07:53 hours to 11:50 hours) for the late morning feeding. Since infants and mothers already have set feeding patterns, the time of feeding was not standardized and thus varied as indicated here.

### Salivary Assay Findings

Oxytocin levels were detected in all samples ( $n = 196$ ) via EIA, ranging from 6.44 pg/ml to 61.05 pg/ml over the course of the study and showed a similar repeating pattern between

Day 1 and Day 2 and across each breast feeding cycle. All statistics are reported below as mean  $\pm$  standard errors (e.g. 35.43  $\pm$  4.16 pg/ml).

### Oxytocin Patterns Across the Breast Feeding Cycle

A separate repeated measures ANOVA was conducted for Day 1 and for Day 2. A similar pattern of change in oxytocin was identified in both Day 1 and Day 2 samples. Oxytocin levels were the highest before feeding (Day 1: 40.48  $\pm$  3.09 pg/ml, Day 2: 35.98  $\pm$  2.89 pg/ml), decreased to the lowest level at the initiation of feeding (Day 1: 16.21  $\pm$  1.98 pg/ml, Day 2: 16.97  $\pm$  1.11 pg/ml), and followed by a moderate increase after feeding (Day 1: 24.43  $\pm$  1.51 pg/ml, Day 2: 21.78  $\pm$  1.37 pg/ml). The change in oxytocin during a breast feeding cycle was statistically significant [Day 1:  $F(2, 31) = 18.94, p < .01$ ; Day 2:  $F(2, 29) = 20.87, p < .01$ ]. The pattern of change in oxytocin did not differ across the three times of feeding in both Day 1 and Day 2 samples. Thus Day 1 and Day 2 samples were combined for further analysis.

A repeated measures ANOVA was conducted to examine if there was a pattern of change in oxytocin levels during a breast feeding cycle; and if the pattern of change in oxytocin during the feeding cycle differed by the time of feeding. The change in oxytocin from the first through the third sample collection was statistically significant [ $F(2,27) = 54.56, p < .05$ ]. Significant differences were identified across the breast feeding cycle between the before feeding vs. initiation of feeding, and between the before feeding vs. 30 minutes after feeding. There was no statistically significant interaction between the change in oxytocin during the feeding cycle and the time of feeding (Figure). Thus, this pattern of change in oxytocin did not differ by the time of day.

The final analysis considered the three breast feeding cycles with time of feeding and Days 1 and 2 combined. A repeated measures ANOVA yielded a statistically significant change in oxytocin from the first through the third sample collection [ $F(2,62) = 39.05, p < .001$ ]. Oxytocin levels were the highest before feeding (38.30  $\pm$  2.12 pg/ml), followed by a decrease at the initiation of feeding (16.58  $\pm$  1.14 pg/ml), and an increase at 30 minutes after the feeding (23.15  $\pm$  1.03 pg/ml). Post-hoc paired *t*-test (two-tailed) analyses using a Bonferroni adjustment (0.05/3) yielded significant differences between before feeding vs. initiation of feeding [ $t(63) = 8.800, p < .017$  (two-tailed)], initiation of feeding vs. after feeding [ $t(63) = -3.703, p < .017$  (two-tailed)], and before feeding vs. after feeding [ $t(63) = 7.218, p < .017$  (two-tailed)].

### Discussion

Using the methods described here, oxytocin levels as assessed by EIA, showed a consistent pattern within and across feeding cycles, thus confirming that oxytocin can be detected in human saliva. The highest oxytocin levels were identified before feeding. At the initiation of feeding, oxytocin was significantly lower. By 30 minutes post completion of feeding, oxytocin significantly increased, although it did not reach the pre-feeding level. This pattern was statistically significant and highly consistent regardless of the time of feeding.

The present findings suggest that oxytocin increased in anticipation of breast feeding. The present data support and extend previous research measuring plasma oxytocin, which suggested that maternal anticipation of breastfeeding is associated with a rise in plasma oxytocin, especially in association with a baby's restlessness or crying (McNeilly, Robinson, Houston, & Howie, 1983). An early study of oxytocin release in blood during feeding demonstrated that only one of ten women experienced an anticipatory release of serum oxytocin while seven women exhibited a rise in oxytocin during breastfeeding in the early postpartum period. However, in the latter study, babies were brought to their mothers only

one minute prior to initiation of breastfeeding (Lucas, Drewett, & Mitchell, 1980). In contrast, another researcher measured an anticipatory rise of oxytocin while the baby was with the mother for ten minutes before breastfeeding; this research was conducted during the first three postpartum months. In the latter study serum oxytocin levels of all ten women increased 3 to 10 minutes prior to initiation of breastfeeding in direct response to the baby's prefeeding behaviors (McNeilly et al., 1983).

The present data add to previous research in animal models and in women which suggests that the release of oxytocin can become conditioned to cues associated with breast feeding or the infant. Central and peripheral oxytocin have been found to increase with initiation of lactation in Rhesus monkeys (Amico et al., 1990). In one study of five women, breast pump stimulation increased plasma oxytocin levels within 1 minute of initiation of pumping (Leake, Waters, Rubin, Buster, & Fisher, 1983). In the early postpartum period, breast massage yielded a significant and steady increase of oxytocin while breastfeeding produced an increase of oxytocin in a pulsatile pattern (Yokoyama, Ueda, Irahara, & Aono, 1994). Oxytocin levels have also increased with pumping in vulnerable populations, e.g. mothers of premature infants (Chatterton et al., 2000).

We had initially hypothesized that the highest levels of oxytocin would be found at the time period closest to actual breast contact. However, the data from the present study were highly consistent and left no question that oxytocin was highest during the pre-feeding period. This study was useful for testing the biological validity of measures of salivary oxytocin. However, the somewhat artificial constraints on the mother's response to her infant likely accounted for the pattern of oxytocin release that was detected here.

Future research using this assay may allow us a better understanding of the pattern of oxytocin release in different populations, or under conditions in which samples are taken at more frequent intervals and so forth. Animal research on lactation has suggested that oxytocin patterns in cerebral spinal fluid (CSF) may be independent from patterns in plasma (Amico et al., 1990). However, studies in rats using microdialysis suggest that peripheral and central release of oxytocin are to some extent coordinated (Landgraf and Neumann, 2004). Further research in humans is needed to compare the timing of the release of oxytocin in samples taken from different sources including CSF, saliva, urine and plasma. For example, in comparison to plasma, salivary samples may reflect a more delayed or more rapid response to changes in oxytocin release.

In a pilot study we measured by EIA both salivary and blood levels of oxytocin as a function of massage in young men (Bello, 2007; Carter et al., 2007). Oxytocin was detected in those samples with levels ranging between approximately 1–2 pg/ml in saliva and 150–250 pg/ml in blood. Following massage, there was a reliable yet relatively small increase in oxytocin ( $p < .05$ ). Furthermore, oxytocin increased significantly after massage in both saliva and blood, although controls for repeated testing were not available. More recently, using a variant of EIA assay described in Carter et al. (2007), Holt-Lunstad, Birmingham, & Light (2008) measured salivary oxytocin following a warm touch enhancement intervention. The authors retrieved a pre and post plasma oxytocin sample by a single-stick venipuncture following five minutes of "close warm couple contact" (p. 978), and they found no significant difference in plasma oxytocin levels at baseline. They also had the participants retrieve a saliva sample at home once during week one and twice during week four on a day when the intervention was performed. In the Holt-Lunstad study, the salivary oxytocin samples retrieved at all three time points were significantly higher in the intervention versus the control group ( $p < .01$ ). Consistent with the values described here, in that study mean levels of oxytocin were 14.73 pg/ml and 6.52 pg/ml at week one and 16.22 pg/ml and 6.92 pg/ml at week four for the intervention and control group, respectively.

One recent published study reported that meaningful levels of oxytocin could not be measured in saliva by EIA (Horvat-Gordon et al., 2005). There are several methodological differences between our studies and theirs, the most important of which is probably the fact that we dried (in the cold) and re-suspended all samples, concentrating samples fourfold to produce levels within the range of the EIA. In addition, the unsuccessful study also used extraction in some of their attempts, which might have reduced the amount of measurable peptide. Since this is one of the first reports of human salivary oxytocin assays, there are many unknowns. All samples were refrigerated immediately after collection and stored at  $-80^{\circ}\text{C}$  within 4 hours after retrieval (and within 24 hours of the first sample collection). Further research is needed to document the optimal preparation and storage of samples for assays, i.e. necessity of immediate refrigeration or freezing, length of time samples can remain unrefrigerated, and length of frozen storage time of samples prior to assay. Additional information regarding stimulation of saliva (gum, citric acid, sugar) and assays is also required.

### Limitations

This research was conducted in the home, a naturalistic setting, and thus mothers and babies had different routines for breastfeeding. There was a large variation in the time of feeding, especially for the first morning feeding. There was also variation in the time of saliva collection within each feeding cycle. Furthermore, because the mothers were retrieving the samples in the home, we relied on self-report. To more precisely confirm correct saliva retrieval, further research should evaluate the length of time it took for collection of each sample and the exact timing of the saliva collection in relationship to the starting of the feeding. An approach to confirm correct retrieval would be to have the mothers collect at least one sample in the lab or in the home under observation. This study was performed with 11 participants. Future research should examine oxytocin levels in a larger sample of lactating women. Furthermore, future research is needed to assess the influence of a circadian rhythm on oxytocin levels by utilizing a narrower infant age range, and retrieving more detail as to night and day light and sleep patterns.

### Conclusion

The potential for noninvasive measurements of oxytocin offers a new approach to understanding the role of oxytocin in health and illness. Having a reliable measurement of oxytocin via saliva will allow a better understanding of the factors regulating oxytocin release, and may provide researchers the opportunity to retrieve samples throughout a participant's daily activities and in a less stressful environment such as the participant's home. Further research is warranted to document the relationship between measures of the salivary oxytocin assay and those taken in blood and urine. However, this study offers further support for the hypothesis that oxytocin is detected in saliva during lactation at levels that change consistently, and that oxytocin can be measured if the assay in use has sufficient sensitivity.

### Acknowledgments

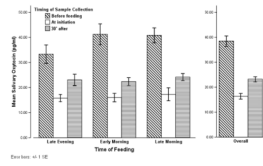
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**Figure.**  
Change in salivary oxytocin during the three feeding cycles (N=11)