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Oxytocin is a nine-amino acid peptide hormone and/or neuropeptide that has proposed mechanisms related to diminishing the effects of physical, psychological, and psychosocial stress. Exercise is often used in clinical trials to quantify stress because it is easily reproducible between subjects using relative VO_2 intensities. However, trials examining plasma oxytocin in response to physical stress within exercise have shown mixed results possibly related to the varied measurement techniques. The purpose of this study is to measure plasma oxytocin changes in response to steady state exercise and graded maximal exercise and to determine if a relationship exists. Procedures were utilized to release oxytocin from the protein binding within blood and measure the oxytocin concentration through a high-performance liquid chromatography coupled to a mass spectrometer. Difficulties in the chromatography detection sensitivity forced the present study to also use an ELISA to determine plasma oxytocin concentrations. Plasma oxytocin concentrations were consistently too low to detect even after sizable spiking for both measurement techniques. Mean resting concentration derived from the ELISA was 371.75 ± 215.10 pg/ml. There was no significant difference in concentration following either exercise stimulus. These results suggest that the extraction technique utilized with the ELISA kit failed to separate protein binding from oxytocin prior to analysis.

THE EFFECTS OF ACUTE EXERCISE ON PLASMA OXYTOCIN

by

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APPROVAL PAGE

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CHAPTER I

INTRODUCTION

With the ever-increasing prevalence of documented anxiety disorders, it is becoming more important to better understand these disorders. Researching proposed biomarkers associated with anxiety disorders may help us better diagnose and treat anxiety. In 2013, the total healthcare cost for mental disorders was $201 billion, which was higher than many other diseases and conditions (Roehrig et al. 2016). Of all the psychiatric conditions, anxiety disorders are the most common and have a lifetime prevalence close to 30% in the United States (Kessler et al. 2008, Naja et al. 2017). Anxiety disorders are typically characterized by more than the normal amount of worry and fear. Based on the NIMH website, "People with generalized anxiety disorder display excessive anxiety or worry for months and face several anxiety-related symptoms." These symptoms include: restlessness or feeling wound-up or on edge, being easily fatigued, difficulty concentrating or having their minds go blank, irritability, muscle tension, difficulty controlling their worry, and sleep problems (difficulty falling or staying asleep or restless, unsatisfying sleep). One of the risk factors associated with social anxiety disorders is elevated cortisol levels in the afternoon and higher resting plasma oxytocin concentrations.

Often, therapy is suggested to help with anxiety disorders. This may include talk therapy, cognitive behavioral therapy, support groups, stress-management techniques, and medication. These interventions often enable the person to cope with the disorder but typically does not cure the person of the disorder. There is some evidence that aerobic exercise has a calming effect in some individuals, but the quality of the results from these studies are not strong enough to support its use as treatment. The 1996 Surgeon General's Report on Physical Activity and Health concluded that feelings of anxiety are reduced with regular physical activity. One of the suggested reasons for aerobic exercise to treat anxiety is related to the effects of various hormonal signals including catecholamines, cortisol, and oxytocin (Naja et al. 2017).

Exercise is often seen as an anxiety and stress-reducing activity; however, it could also be a cause of anxiety and stress if the exercise is too difficult (intense) or unfamiliar. Cortisol is an acute stress related hormone that is induced by activation of the HPA axis and is elevated within the blood in response to intense exercise (Brown et al. 2016). A stress stimulus will result in a cascade of effects within the HPA axis and stimulate the synthesis of cortisol in the adrenal cortex as well as release of catecholamines from the adrenal medulla. The type, intensity and duration of the stress will affect cortisol concentration. Exercise will stimulate cortisol increases in the circulation depending on the intensity and duration of the exercise. Anxiety-related stress could be in relation to the rise in blood cortisol released in response to exercise (Whirledge et al. 2010). Cortisol is released as a result of intense exercise to increase glucose formation (gluconeogenesis) and, thus, provide more carbohydrate fuel for the intense exercise (Whirledge et al. 2010,

Castro-Vale et al. 2016). High blood concentration of this glucocorticoid is correlated with stress, fear, pain, psychological stress, and many different anxiety disorders (Brown et al. 2016, Castro-Vale et al. 2016). In contrast, oxytocin has been theorized to have a suppressive effect on cortisol release and, therefore, may have an anxiolytic effect (Naja et al. 2017).

Oxytocin is a neuropeptide that could change the way anxiety disorders' treatments evolve. This neuropeptide has been reported in multiple studies to increase trust and social bonding and to reduce anxiety in response to stressful situations (Naja et al. 2017). Oxytocin in response to exercise has had mixed results and appears to be related to several factors, such as type and duration of exercise as well as exercise intensity (Hew-Butler et al. 2008). Salivary oxytocin was shown to increase significantly following 10 minutes of running in both men and women with higher levels reported for women (Jong et al. 2016). The exercise increase was similar to or greater than the increases with a standardized Trier Social Stress Test (TSST) in these same individuals. The cortisol response was elevated in these individuals in saliva but was at a later time (Jong et al. 2016). Blood cortisol levels do not significantly elevate until roughly 30 minutes after a stress stimulus. It must be activated by a cascade effect from the hypothalamus and pituitary gland before being synthesized in the adrenal cortex and subsequently released. In contrast, acute changes in oxytocin were reported not to be elevated after a high-intensity steady state run for 60 min but did increase after an ultramarathon (Hew-Butler et al. 2008). The response to a maximal graded exercise test reported a non-significant increase from 1.3 to 2.6 pg/ml in this study. The non-

significant result may possibly be related to a small sample size (n=7), as well as the large variation in oxytocin levels in this study. Another factor which may contribute to the lack of consistency in the literature with oxytocin changes with exercise is the procedures utilized to measure this molecule. In this thesis it has been attempted to follow the procedures by Brandtzaeg et al. to purify the plasma samples (2016).

Purpose

The purpose of this study is to determine the changes of plasma oxytocin in a healthy population in response to a graded exercise test and a moderate intensity 30-minute exercise to understand if plasma oxytocin is related to exercise intensity and or duration.

Objective and Hypotheses

Although oxytocin has been measured in response to social stress, few studies have recorded its response to exercise. Understanding oxytocin concentration's response to exercise may give us a better understanding of the role of exercise in stress reduction. It is hypothesized that oxytocin concentration will significantly increase in response to graded maximal exercise. In contrast, it is hypothesized that 30 minutes of sustained aerobic moderate intensity exercise (70-75% VO_{2max}) will not provide sufficient intensity to induce a significant increase in plasma oxytocin concentration. It is unclear if there will be a relationship between the change in cortisol and the change in oxytocin due to secretion of cortisol occurring later in time post exercise. Salivary oxytocin peaks in concentration about 10-15 minutes after psychological and physical stress stimulus, whereas salivary cortisol does not peak until 35 minutes after stimulus (Jong et al. 2015).

Finally, there is a conflict in the normal resting oxytocin concentration. Depending on the extraction and analysis methods, researches have reported baseline levels as low as 1 pg/ml and as high as 1000 pg/ml. Recently, liquid chromatography and mass spectrometry was used to directly measure plasma oxytocin and reported baseline plasma levels around 600-800 pg/ml. Brandtzaeg et al. 2016 suggests that better extraction of oxytocin is needed. Oxytocin binds strongly to proteins in the blood and requires precise extraction for accurate measurement. Commercial radio immunoassays and enzyme immunoassays kits that include extraction sometimes fail to depict true plasma oxytocin levels. Some showed levels two magnitudes higher than normal (McCullough et al. 2013). Although some RIAs possess lower limits of quantification (LLOQ) as low as 0.49 pg/ml, RIAs being an indirect measure opens itself up to disruption in the form of pH variation and extra binding to macromolecules in biological samples (Zhang et al. 2011). High pressure liquid chromatography paired with mass spectrometry may alleviate some of the inaccuracies of the above-mentioned methods. Quantitative measurements of endogenous oxytocin are challenging because of the low concentration that may be found in human plasma. While few methods of HPLC-MS analysis of oxytocin are available, some have shown LLOQs as low as 1.00 pg/ml (Zhang 2011). This approach may be the best for determination of basal oxytocin levels with high sensitivity. Liquid chromatography results were compared with enzyme linked immunosorbent assay (ELISA) results to determine validity of the two procedures.

Therefore, this thesis hopes to report the baseline levels of a healthy young cohort at rest and to determine if oxytocin in this cohort will be elevated immediately after these two types of acute exercise.

Assumptions and Limitations

Subjects were apparently healthy and had not exercised for at least 24 hours prior to testing. The subjects came into the lab after an overnight fast and were adequately hydrated. Subjects were non-tobacco users for at least 6 months prior to the study, had normal resting blood pressure and were not abnormally anxious.

All subjects had normal resting blood pressures and were not obese based on seven site skin fold. Only apparently young subjects (18-35 years of age) were recruited, so the results may not reflect what may occur in older individuals. This is a small cohort of subjects and, therefore, may not reflect what can occur in all subjects. All subjects reported in a post-absorptive state and thus these values may not be similar in a fed state.

CHAPTER II

REVIEW OF THE LITERATURE

General Oxytocin Information

Oxytocin (OXT) is a neuropeptide as well as a peptide hormone that plays a critical role in social recognition, anxiety, and childbirth (Naja et al. 2017). Although OXT has been well researched in childbirth, there has been limited research studies observing the effect of physical stress on OXT response (Brown el al. 2016, Hew-Butler et al. 2010). Oxytocin is synthesized in the magnocellular neurosecretory cell of the paraventricular nucleus and the supraoptic nuclei in the hypothalamus. Oxytocin is secreted from the posterior pituitary into the hypothalamic pituitary adrenal axis (HPA axis). As the name implies, this axis is the collection of the following three endocrine glands: the hypothalamus, the anterior and posterior lobes of the pituitary gland, and the adrenal cortex. Oxytocin has also been shown to be secreted within the brain and from the ovaries and testes. The common feedback loop that occurs in this axis is the regulation of the glucocorticoid cortisol (Castro-Vale et al. 2016). Oxytocin is hypothesized to have a role in this regulatory process, but the exact function is unclear. It is currently believed that OXT may have an inhibitory effect on corticotropin-releasing hormone (CRH) to limit the secretion of cortisol and reduce the stress response (Naja et al. 2017). Thus, OXT has an important anxiolytic regulatory effect on the HPA axis.

OXT may be regulated in part by catecholamines. The adrenergic system has dual control over oxytocin with α-receptor activation increasing secretion while β-receptor activation demonstrating an inhibitory effect on secretion (Song et al. 1988).

The oxytocin effect is determined by its binding and activation of its receptor the oxytocin receptor (OXTR). OXTR is coupled to a G protein receptor located in the membrane to work within several brain regions. Although OXTR mapping has not been completely done in humans, there has been a good deal of research in rodents and primates. In these animals, OXTR was located in the spinal cord, brainstem, hypothalamus, amygdala, accumbens nucleus, hippocampus, and anterior cingulate cortex (Naja et al. 2017). These brain regions with OXTR present are involved in social reward, emotion and memory. Recently, studies observing the human brain during intranasal OXT administration observed reduced activation of the amygdala (Naja et al. 2017, Mottolese et al. 2014). This introduces the idea that the amygdala is largely involved in the role of oxytocin and stress regulation with a possible interaction with serotonin (Mottolese et al. 2014).

Oxytocinase is an enzyme in the aminopeptidase family that is responsible for the metabolism of oxytocin (Tsujimoto et al. 2004). Oxytocinase deactivates oxytocin by cleaving the peptide bond between the N-terminal cysteine and the adjacent tyrosine residues. Because of its similar structure and location, vasopressin is also easily degraded by oxytocinase. Enzyme expression of oxytocinase could have effects on the sensitivity and effect of oxytocin and vasopressin in the human body. Oxytocin has a half-life of

about 7-15 minutes in rats depending on the concentration with longer times with very high doses (Morin et al. 2008).

Factors Affecting Oxytocin Synthesis and Release

Biosynthesis of oxytocin begins with the synthesis of an inactive precursor protein, which gets progressively hydrolyzed and cleaved into smaller parts. Within the paraventricular nucleus or supraoptic nucleus, which lie within the hypothalamus, oxytocin is hydrolyzed one final time to its active nonapeptide form by the enzyme Peptidyl-glycine alpha-amidating monooxygenase (PaM). Although it is synthesized in both regions, oxytocin is more prevalent in the paraventricular nuclei. PaM's function is dependent on a binding cofactor of sodium ascorbate or vitamin C. This hydrolyzation process also yields neurophysin I, the carrier protein for oxytocin (Landgraf et al. 1981). The correlation of plasma concentration of both neurophysin I and oxytocin supports this carrier synthesis theory (Brownstein et al. 1980). Both levels will increase in response to dehydration and lactation (Dax et al. 1978).

Oxytocin Anxiolytic Effects

It is currently unknown what the specific mechanisms are that regulate the secretion of OXT outside its role in childbirth. There are, however, distinct rises in OXT blood plasma concentration following sexual stimulation, childbirth, anxiety, and hyperosmolarity (Naja et al. 2017, Brown et al. 2016, Hew-Butler et al. 2010). Functional magnetic resonance imaging (fMRI) has been used in humans to determine behavioral and cognitive responses to OXT. Observing the brain after OXT administration has

shown increased activity in the cortical and subcortical regions of the cerebral cortex, temporal lobes, amygdala, and prefrontal cortex (Naja et al. 2017).

Both oxytocin and serotonin are significant neuromodulators that influence social interaction, stress, and anxiety. Recent animal studies have reported a direct anatomical link between the two brain regions carrying these transmitters. Serotonergic fibers originating in the dorsal and medial raphe nuclei of the brainstem connect to the paraventricular nuclei and the supraoptic nuclei (Mottolese et al. 2014). Further investigation has shown oxytocin receptors in the raphe nuclei, the place of serotonin synthesis. Oxytocin infusions in animals have noted increased serotonin concentration and infusion of serotonin increases oxytocin (Mottolese et al. 2014). Therefore, both neuromodulators work in tandem to react to varying social stimuli, such as fear and social bonding.

Anxiety Disorders and Oxytocin

Brain oxytocin activity can be used to categorize emotional and social behaviors (Neumann et al. 2012). High anxiety levels can be related to an imbalance of OXT systems. This can be a result of reduced OXT gene expression, reduced central nervous system OXT release or availability, low OXTR expression, or an imbalance of AVP and OXT (Neumann et al. 2012, Naja et al. 2017). Contrary to the evidence of OXT's anxiolytic effects, many studies have shown increased peripheral OXT is related to increased anxiety and distress (Brown et al. 2016). High levels of plasma OXT were correlated with reduced depressive symptoms in women with psychological stress (Zelkowitz et al, 2014). They suggested that the plasma OXT was there to attenuate or

mitigate the psychological and physiological stress and this is supported by Heinrich et al. 2003. In male subjects with post-traumatic stress disorder, a lower concentration of salivary OXT was observed compared to trauma control subjects (Naja et al. 2017). This is in line with many other animal studies where plasma, salivary fluid, and cerebral spinal fluid OXT concentrations were lower in general anxiety disorders (GAD) subjects compared to normal subjects (Jong et al. 2015).

Oxytocin and arginine vasopressin (AVP) work together to regulate anxiety as well as regulating fluid volume. It is believed both neurotransmitters are released in response to anxiety and have opposite effects (Neumann et al. 2012). Acting in the amygdala, oxytocin modulates neuronal functions to reduce emotional responsiveness and stress. Oxytocin has mostly been studied in response to psychological stress as opposed to physical stress. Arginine vasopressin is released in tandem with OXT during both psychological and physical stress inducing situations. AVP typically has an anxiogenic response, and high expression of AVP receptors in brain regions is linked to increased social anxiety (Neumann et al. 2012). With exercise that results in changes in plasma volume loss, AVP will be elevated to try to conserve water loss through the kidneys.

Relationship of Cortisol and Stress

In response to a stressful or traumatic event, the paraventricular nucleus in the hypothalamus releases corticotrophin-releasing hormone (CRH) and vasopressin, both of stimulate adrenocorticotropic hormone (ACTH) to be secreted from the anterior pituitary (Raymond et al. 2017). This cascade effect in the HPA axis results in ACTH stimulation

of adrenal cortex synthesis and secretion of cortisol. This cortisol contributes to the mobilization of the body's energy sources in preparation for action. In turn, the cortisol rise will inhibit CRH and ACTH to create a negative feedback loop.

Cortisol release and its subsequent effects play a critical role in stress regulation, and cortisol is commonly used as a stress biomarker (Raymond et al. 2017). Cortisol prepares the body for stress and influences many different systems of the body. Because of its lipid-solubility, it passes the blood brain barrier easily to affect a multitude of brain regions. These include but are not limited to the following: the amygdala, the hippocampus, the prefrontal cortex, and the anterior pituitary.

Oxytocin Effect on Cortisol

Pharmaceutical stress treatments using intranasal and intravenous OXT administration have been shown to reduce the effect of stress (Naja et al. 2017). There is evidence that exogenous oxytocin can inhibit CRH in the HPA axis while arginine vasopressin enhances CRH release (Raymond et al. 2017). Decreased plasma cortisol was noted following intravenous and intranasal oxytocin administration in response to stress (Naja et al. 2017, Cardoso et al. 2013). Although this information may lead to a more fine-grained understanding of the mechanism for OXT release, it has not been unanimously reported in human studies. It also appears oxytocin has a dose-dependent response for cortisol (Cardoso et al. 2013). Intranasal administration of oxytocin during vigorous exercise only had a reduced cortisol release with a 24IU dose. Meanwhile, subjects that received a 48IU dose had similar cortisol concentrations to control subjects

(Cardoso et al. 2013). It is unclear what the most effective dose of oxytocin may be for stress reduction.

fMRI scans of the human brain have shown that both OXT and cortisol have receptors in a number of the same brain regions, including the amygdala, hippocampus, prefrontal cortex, and the anterior pituitary (Raymond et al. 2017, Naja et al. 2017, Mottolese et al. 2014). In the amygdala, the stress response initiated by cortisol elicits a strong emotional response, such as fear or anxiety. The oxytocin and serotonin interaction within the amygdala lessens this emotional response (Mottolese et al. 2014). An excess of cortisol can impair the hippocampus' ability to encode and recall information. The result of this is an impaired ability to recall information during stressful situations. At the same time, the emotional response enhances memory consolidation to improve the memory of the stressful event after the fact (Payne et al. 2007). Oxytocin's role in these brain regions is largely unknown. However, oxytocin administration has resulted in attenuated emotional response and improved memory during stress (Neumann et al. 2008).

Hydration's Role in Peripheral Oxytocin

Arginine vasopressin (AVP) and aldosterone are the principle hormones responsible for fluid balance. These hormones are secreted in response to increased blood osmolarity and decreased blood volume. Vasopressin stimulates vasopressin receptor 2 (V_2) receptors in the kidney tubule to promote water retention (Hew-Butler et al. 2010). Aldosterone stimulates mineralocorticoid receptors in the distal kidney tubules to increase sodium reabsorption. Vasopressin secretion is very sensitive to osmolarity with significant AVP release following only a 1-2% change in blood osmolarity (Hew-Butler

et al. 2010). The AVP response to exercise has been well documented, but changes in plasma osmolarity during exercise are only partially the result of a direct effect of AVP (Hew-Butler et al. 2010). During exercise, aldosterone is activated at a lower exercise intensity than AVP. During times when there is increased intensity, both AVP and aldosterone work together to retain water as plasma volume is lost.

Oxytocin and AVP are synthesized in the same regions and have very similar structure. Both hormones only differ by two amino acid groups. With such similarities, the two hormones tend to have an overlap in receptor activation. Oxytocin may be responsible for some of the differences in osmolarity during exercise. OXT can stimulate the V_2 receptors on the kidney similarly to AVP but needs a 100-fold higher concentration to have the same effect as AVP (Hew-Butler et al. 2010). Therefore, OXT is likely not a major factor for fluid balance as the osmolarity concentration within the circulation does not increase to that extent, even with exercise that demonstrates osmolarity increases. However, fluid balance shifts may stimulate oxytocin release in similar manners as vasopressin release. This needs to be addressed in research focused on oxytocin in situations where subjects may experience fluid loss.

Oxytocin and Acute Exercise

The effect of acute exercise on oxytocin is under-researched. The few studies available currently do not show much change in plasma OXT concentrations in response to exercise (Chicharro et al. 2001). Jong et al. 2015 observed salivary oxytocin in response to psychosocial stress, sexual stimulation, and physical exercise. All three stimuli showed similar responses with peaks at the end of stimulation and a gradual recovery over time. The physical exercise was running at a self-selected pace for 10 minutes. Salivary oxytocin rose from about 2 pg/ml resting to close to 5 pg/ml directly post-run. Testing was done at home, and participants collected their own saliva.

Hew-Butler et al. 2008 showed no change in plasma oxytocin following a graded treadmill max test and a 60-minute steady state at 60% peak running speed. However, a significant increase in plasma oxytocin was observed after an ultramarathon, 1.5 pg/ml prior ultramarathon and 3.5 pg/ml post. It was hypothesized that this increase was not due to the exercise stimulus but was, instead, from the extreme fluid loss. This study had only seven participants, and each of them were highly-trained endurance runners. The endocrine response to this physical exercise may be attenuated in the endurance runners compared to the normal population.

Chicharro et al. 2001 explored the differences that training status had on plasma oxytocin's response to exercise. They used a cycling ramp protocol and measured plasma oxytocin at rest, below the lactate threshold, above the threshold, and at VO_2max. They reported no plasma OXT change in both groups to the various intensities. They did observe lower OXT concentrations in the trained group compared to the untrained, about

10 pg/ml and 18 pg/ml respectively. These values are slightly higher than the previously established basal levels of 1-10 pg/ml (McCullough et al. 2013, Zhang et al. 2011) and suggest that preparation of the plasma samples may be a factor as to why exercise had no influence on the OXT concentration.

Altemus et al. 2001 examined the differences in female sex hormone, oxytocin, and vasopressin responses to exercise and compared these responses in the varying phases of the menstrual cycle. An exercise test with 5-minute stages at 50%, 70%, and 90% VO_2 max was utilized. No significant change in OXT was noted immediately post-exercise nor during a 40-minute recovery.

Table 1. Summary of Acute Exercise Training Effect on Oxytocin

Study	Subjects	Exercise Modality	Pre-Ex [OXT] (pg/ml)	Post-Ex [OXT] (pg/ml)	Assay	Tissue
Chicharro et al. 2001	Trained vs Untrained cyclists	Ramp cycling till exhaustion	Trained ~10 Untrained ~18	Trained ~10 Untrained ~18	RIA	Plasma
Hew-Butler et al. 2008	Trained Endurance Runners	Graded treadmill VO_2 max test	1.3 (0.2)	2.6 (0.7)	RIA	Plasma
Hew-Butler et al. 2008	Trained Endurance Runners	60 minute steady state at 60% VO_2 max	1.1 (0.3)	1.5 (0.1)	RIA	Plasma
Hew-Butler et al. 2008	Trained Endurance Runners	56km ultramarathon	1.5 (0.2)	3.5 (0.5)*	RIA	Plasma
Jong et al. 2016	Healthy Adults	10 minutes of self-selected moderate running	~2.0	~5.0*	RIA	Saliva

* Denotes significantly different from pre-exercise oxytocin $p<0.05$.

Oxytocin and Chronic Exercise Training

Very little is known about how training status influences oxytocin dynamics in humans. The lack of evidence makes it difficult to draw any conclusions on the implications of endurance training. In the only study that was found that compared trained cyclist (n=12) versus untrained athletes (n=10), trained subjects had significantly lower plasma OXT levels at rest (10 pg/dl), during exercise below lactate threshold, during exercise above lactate threshold, and at maximal exercise compared to untrained (17-18 pg/dl) (Chicharro et al. 2001). Both groups showed no change in OXT with exercise. OXT was measured using a radioimmunoassay kit by Phoenix Pharmaceutical after passing samples through a Sep-Pak C18 cartridge. The authors attributed the reduced OXT in the trained group to an attenuated release of substrates from the hypothalamus. They suggested this might be related to a lessened need for vasopressin to regulate arterioles and fluid balance during exercise. There has been evidence that chronic exercise upregulated OXT gene expression and content in the hypothalamus of rats (Gutkowska et al. 2007).

Oxytocin Gender Differences

Females may have higher levels of oxytocin during rest and have twice the prevalence of anxiety disorders as their male counterparts (Naja et al. 2017). Males and females have complex differences in oxytocin secretion and function. Some observations of both humans and animals studies noted different brain regions activated in responses to the same stimuli between the sexes. Also, social dynamics with oxytocin exposure differed between the sexes in rodents (Naja et al. 2017). These differences are poorly

understood, but breakthroughs could change how anxiety disorders are treated for these two genders. One interesting difference that may have a large effect is the expression of OXT peptide synthase on the OXTR. Females have more OXT peptide synthase and more OXTR in the amygdala due to upregulation via estrogen (Naja et al. 2017, Neumann et al. 2012).

Measurement of Oxytocin

Recent studies have shown evidence that traditional methods of measurement of oxytocin have varied selectivity and may report falsely high and low values (Brandtzaeg et al. 2016, Brown et al. 2016). It is hypothesized that this issue arises from oxytocin binding strongly to proteins in plasma and serum (Brandtzaeg et al. 2016). To avoid this issue, a protein precipitation and an extraction technique were implemented by these researchers. This process extracts oxytocin from serum or plasma after removing it from the protein and evaporates it to dryness and then reconstitutes the OXT prior to assay. HPLC tied to mass spectrometry was reported to have the highest sensitivity with lower limits of quantification (LLOQ) as low as 1.0 pg/ml (Brandtzaeg et al. 2016, Zhang et al. 2011). Unextracted plasma samples were compared to the extracted samples. The unextracted samples showed an average of 60 times higher OXT than the extracted samples (Brown et al. 2016). Therefore, it is likely that the reason for the modest change in the results from other studies was the 60 times higher OXT level in those papers as these studies did not extract the OXT from the protein. Extraction of oxytocin may also present issues in quantification analysis. There is a possibility with all extraction

techniques that there is oxytocin lost during the process. Liquid chromatography has excellent selectivity and may be the best method for extraction and quantification.

Radioimmunoassay (RIA) and enzyme immunoassay (EIA) techniques have been observed to have varied sensitivity and validity when determining oxytocin levels in human plasma. These two techniques also require protein extraction for accurate OXT analysis. Radioimmunoassay methods may have difficulties detecting OXT concentrations below 10 pg/ml (McCullough et al. 2013). This issue persists in most enzyme linked immunosorbent assay kits with sensitivities around 17 pg/ml (Arbor Assay). The inability to detect plasma levels below 10 pg/ml makes it challenging for these techniques to be used in human plasma where basal levels have been reported to be as low as 1-10 pg/ml (McCullough et al. 2013).

Table 2. Summary of Baseline Oxytocin Measurement

Study	Subjects	Oxytocin Concentration (pg/ml)	Extracted	Assay	Tissue
Altemus et al. 2001	Health Women	1.66 (0.10)	Yes	RIA	Plasma
Chicharro et al. 2001	Trained vs Untrained cyclists	Trained ~10 Untrained ~18	Yes	RIA	Plasma
Hew-Butler et al. 2008	Trained Endurance Runners	1.3 (0.2)	Yes	RIA	Plasma
Jong et al. 2016	Healthy Adults	~2.0	Yes	RIA	Saliva
Landgraf et al. 1982	Healthy Adult Men	~4.1 (2.7)	Yes	RIA	Plasma
Zhang et al. 2011	Health Adults	2.58 (0.275)	Yes	LC-MS	Plasma
Bello Et al. 2008	Healthy Men	92.3	No	EIA	Plasma

Values shown are mean followed by standard deviation in parentheses. Table 2 presents basal or resting oxytocin concentration in various studies in human plasma or saliva.

CHAPTER III

METHODS

Subjects

The study protocol for J.D. Waller's thesis was reviewed by IRB and approved. The plasma samples to be utilized to determine the oxytocin levels were obtained in this study and stored in -80°C freezer. Subjects were given ample time to read and ask questions prior to signing the informed consent on their first visit. Fifteen individuals were approved to be subjects. They completed the screening and were non-tobacco users and not taking medications or supplements that would influence metabolism, insulin, and hydration status for at least 6 months. Subjects were instructed to maintain their normal diet throughout the study. The subjects were normotensive based on their resting blood pressure. The participants reported to the laboratory (6-9 a.m.) after an overnight fast and had not exercised for at least 24 hrs. They were all given water to drink to prevent dehydration when they arrived and during the submaximal exercise.

Upon arrival to the laboratory for each visit the subject was seated and rested for a minimum of 15 minutes. The first visit was the VO_2max treatment as this had to proceed the submaximal visit. A heart rate (HR) monitor was placed around their chest (Polar monitor) and captured their resting HR. Subjects, then, had a resting blood sample taken from an antecubital vein. Subjects rested at least another 5 minutes prior to the graded treadmill exercise and were given a water bottle to drink prior to exercise.

Maximal Treadmill Graded Exercise

Subjects had a 5 min warm-up on the treadmill until their HR (Polar monitor) reached ~130 bpm. Subjects continuously breathed through a mouthpiece connected to a one-way valve which enabled continuous analysis of their expired gases. Subjects wore a nose clip which prevented them from breathing through their nose. Thereafter there was an increase of 2.5% grade each minute until reaching VO_2 max or volitional fatigue. Expired gases were collected and analyzed by a ParvoMedics TrueOne 2400 analyzer system (ParvoMedics, Sandy, UT, USA) calibrated to known gases. Ratings of perceived exertion (RPE) were recorded each minute (Noble et al. 1983). All subjects satisfied at least four of the five required criteria for a true VO_2max (Midgley et al. 2007; Pescatello et al. 2014). Immediately after the test (1-2 minutes) another blood sample was taken from an antecubital vein.

Submaximal Treadmill Exercise

Subjects reported back to the laboratory at the same time in a post-absorptive state with at least 3 days between visits but no longer than 14 days. As before, they rested for at least 15 minutes prior to having a blood sample obtained at rest. They again had a polar HR monitor placed around their chest. Subjects were given a 3-5 min warm-up on the treadmill. HR was continually monitored during the 70-75% VO_2max exercise (Polar monitor) and recorded every 5 minutes. VO_2 was confirmed in the initial 5 minutes and 1 minute every 5 minutes during the test to ensure proper intensity. Subject RPE was obtained every 5 minutes to further ensure appropriate subjective intensity was

maintained. Subjects were given water every 2 minutes. Immediately after the test a post-exercise blood sample was obtained within 1-2 minutes of cessation of exercise.

Study Design

A repeated measure, 2 (2 exercise conditions [VO_{2max} and continuous submax]) x 2 (2 time points [Pre, immediately post]) design was used for this study. After a preliminary screening, subjects performed the first exercise session [VO_{2max}] by necessity to enable % workload to be determined. The submaximal workload visit occurred within seven days and at the same time as the previous visit in a post-absorptive state.

Blood Collection and Treatment

Subject rested at least 15 minutes before blood was obtained from an antecubital vein prior to any intervention. Blood was drawn into vacutainer EDTA tubes. Hematocrit (Hct) was determined by taking <25µl blood drawn into micro-hematocrit capillary tubes, sealed and centrifuged (micro centrifuge at 3000 rpm 5 min), and volume ratio determined (triplicate). Blood was immediately centrifuged (Beckman Allegra) for 10 minutes at 3000 rpm (4°C). Plasma was aliquoted into microtubes and placed in a -80°C freezer until analyzed.

Sampling and Assays

Plasma samples were extracted prior to analysis via a Waters Acquity Ultra Performance Liquid Chromatography (UPLC) coupled to a Thermo Fisher Q Exactive Plus High-Resolution Mass Spectrometer (HRMS) system using techniques described in Brandtzaeg, *et al.* 2016. This process uses a protein precipitation to extract OXT from the protein within plasma samples. 5µl of 0.5mM dithiothreitol were added to each sample

for reduction of disulfide bonds, and 15µl of 0.5mM iodoacetamide were added to each sample for alkylation. The samples were vortexed and then placed in a heat block at 37°C for 45 minutes. They were then allowed to cool and then the iodoacetamide was added in darkness for 20 min. Then 700µl of 80% LC-MS grade acetonitrile (ACN) was added to help with protein precipitation. The samples were vortexed and then placed into a micro-centrifuge at 12,000 rcf for 20 minutes. Supernatants (900µl) were removed and passed through 0.22 µm polypro filters to remove any large particles. These samples were all spiked with 10µl of 10ng/ml OXT standard (100 pg) (Sigma-Aldrich). All samples were then placed in a speed vacuum system at 45°C and centrifuged until dryness. The dried samples then were stored in -80°C until analyzed. They were later reconstituted in 100µl of 0.1% formic acid. Most samples required sonication to go into solution. The complete process of the extraction can be found in appendix A. All samples were extracted in duplicate and were analyzed in triplicate through the UPLC-HRMS system.

The UPLC-HRMS -mass spectrometer system used could only detect standards at the ~ 0.2 ng/ml consistently so each sample was spiked with internal standard (IS) as suggested by Brandtzeg et al (2016). Each sample was spiked with 10 µl of [10 ng/ml] standard solution. This was the equivalent to 100 pg added to each sample. The delta concentration will be presented as the concentration of OXT in plasma.

The UPLC used a binary solvent system consisting of water containing 0.1% formic acid and acetonitrile containing 0.1% formic acid. Samples were eluted from a Waters Acuity Pentafluorophenyl (PFP) column at a flow rate of 0.3 mL/min. The PFP

column contains fluorine groups to provide enhanced retention and selectivity. Elution time for OXT was ~2 minutes.

The UPLC-HRMS utilized could not consistently detect the low values of oxytocin in the plasma. Even with the addition of the 100 pg spike this system was unable to assess the peaks for the oxytocin.

Values found from liquid chromatography were compared to results from an enzyme linked immunosorbent assay kit obtained from Arbor Assays. This kit utilizes a similar extraction technique but has the addition of using a C18 Sep-Pak column for added sample separation. 800µl of 1% trifluoracetic acid was combined with 800µl plasma sample and was vortexed completely before being centrifuged. Supernatant was collected and put through the C18 Sep-Pak column after washing the columns with a solution of 60% acetonitrile, 1% trifluoracetic acid, and 39% distilled water. Prior to extraction through the Sep-Pak column one sample was spiked with 100 pg of oxytocin standard. After samples eluted off the column they were transferred to polypropylene tubes and brought to dryness in a speed vac. Samples were reconstituted in 350µl of assay buffer prior to being plated and analyzed. The complete extraction process can be found in Appendix B and protocols for the ELISA can be found in Appendix C. During pilot experiments with this ELISA method the first and second wash after loading plasma onto the Sep-Pak Column were collected and analyzed along with the eluted sample. This was done to measure if any oxytocin was lost during extraction.

Preparation of Standard

97% HPLC grade Oxytocin acetate salt hydrate was purchased from Sigma-Aldrich. Standard was constituted in 0.1% formic acid and a serial dilution was utilized to create a standard curve. The standard curve consisted of points at 1000 pg/ml, 500 pg/ml, 250 pg/ml, 50 pg/ml, 25 pg/ml, 5 pg/ml, and 0 pg/ml. These solutions were stored in -20°C until analyzed by the UPLC-HRMS.

Standards for the enzyme linked immunosorbent assay were done in triplicate with the OXT standard from the Arbor Assay kit. A serial dilution with the standard was performed with assay buffer to make concentrations of 1000 pg/ml, 500 pg/ml, 250 pg/ml, 100 pg/ml, 50 pg/ml, 25 pg/ml, and 12.5 pg/ml. The sensitivity of measurement was noted as 17 pg/ml. Samples were plated in duplicate with 100 µl of reconstituted extracted samples resuspended in assay buffer.

Statistical Analysis

A repeated measures, 2 (two exercise conditions [VO_2max and 70-75% submaximal tests] and 2 times [pre and post] was employed. SPSS v 21 software was utilized to process the data with a significance value set at $P \leq 0.05$ for significance.

CHAPTER IV

RESULTS

Mean age for participants was 22.8 years. The male average age was 22.6 years while the female average age was 22.8 years. They had an average body mass index (BMI) of 23.4 (SD: 3.5) and a mean resting heart rate of 63.4 (SD: 9.2). The mean duration of the max test until exhaustion was 6.9 (SD: 1.1) minutes with an average relative VO_2 max of 51.1 ml/kg/min (SD: 11.1). Male participants' mean body fat percentage calculated by the Siri equation was 10.2% (SD: 5.8%) while the female mean fat percentage was 20.8% (SD: 6.3%). There was no significant change in hematocrit for all subjects between beginning and end of each exercise treatment.

Both the exercise testing modalities stimulated an increase in heart rate within the subjects. During the max test, subjects' heart rates increased in proportion to the workload creating a positive linear relationship to increased workload. All subjects' heart rates increased significantly during the max test with a mean final heart rate of 187.2 ± 1.77 beats per minute. During the steady state test, subjects' heart rate increased linearly then reached steady state. Mean heart rate during the steady state test was 166.22 ± 3.36 beats per minute. Subjects took an average of 4-5 minutes to achieve steady state.

Table 3. Baseline Descriptive Measures

	Total	**Male**	**Female**
n	12	7	5
Age	22.75 (2.96)	22.43 (2.88)	23.20 (3.35)
Weight (kg)	68.56 (13.08)	72.14 (15.11)	63.54 (8.59)
Height (cm)	171.04 (7.53)	174.29 (5.82)	166.5 (7.8)
BMI	23.37 (3.55)	23.66 (3.96)	22.96 (3.27)
RHR (bpm)	65.0 (9.17)	63.43 (9.07)	67.2 (9.86)
Systolic BP (mmHg)	119.67 (9.34)	120.29 (9.83)	118.8 (9.65)
Diastolic BP (mmHg)	77.83 (7.29)	76.57 (8.04)	79.6 (6.54)
MAP	91.78 (7.29)	91.14 (8.51)	92.67 (6.02)
VO_2max (L/min)	3.49 (0.95)	4.02 (0.86)*	2.77 (0.47)*
VO_2max (ml/kg/min)	51.14 (11.07)	56.17 (10.32)	44.09 (8.42)
Max Test Duration (mins)	6.89 (0.3)	7.16 (0.42)	6.52 (0.42)
Siri BF%	15.32% (8.09%)	11.02% (6.11%)*	21.33% (6.84%)*

All Values are as “Mean (Standard Deviation)”. * Significantly different between genders ($p<0.05$).

Table 4. Exercise Variables

	HRmax: VO_2max	**VO_2max Duration (mins)**	**%VO_2max: Submaximal Test**	**Mean HR: Submaximal Test (bpm)**	**Total Work VO_2max (Watts)**	**Total Work Submaximal (Watts)**
Overall	187.2 ± 1.77	6.89 ± 0.3	72.71 ± 1.01	166.22 ± 3.36	876.36 ± 105.54†	1345.41 ± 173.58†
Male	187.86 ± 2.87	7.16 ± 0.42	73.59 ± 1.43	167.19 ± 4.48	1064.5 ± 122.18*†	1558.32 ± 164.58†
Female	186.2 ± 1.77	6.52 ± 0.42	71.48 ± 1.31	164.87 ± 5.63	612.97 ± 111.53*	1047.33 ± 322.35

All values are mean ± standard error of the mean. * denotes a $p<0.05$ significance between the genders. † denotes a $p<0.05$ significance between the exercise modalities.

Table 5. Hematocrit Changes with Exercise

Time	**VO_2max Test**	**70-75% VO_2max**
Pre- Exercise	46.33 ± 2.19	46.91 ± 2.23
Immediately Post Exercise	46.37 ± 1.58	46.40 ± 2.10

Values are presented as percent hematocrit ± standard deviation.

The present study failed to replicate the UPLC-MS results of Brandtzaeg et al. 2016. Despite multiple attempts at extraction and analysis the values were too low to be detected by the mass spectrometer and no data was recorded for these trials. The data from the ELISA is the only oxytocin values that will be reported.

Resting plasma oxytocin was determined to have a mean concentration of 371.75 pg/ml ± 215.10. Neither exercise tests had a significant effect on plasma oxytocin levels. There was no statistical difference comparing pre- and post- concentration for both exercise modalities. Sample duplicates were consistent with no statistical difference between them, $p>0.5$. The standard curve used to compare unknown samples in the ELISA had an excellent coefficient of determination, $r^2=0.996$.

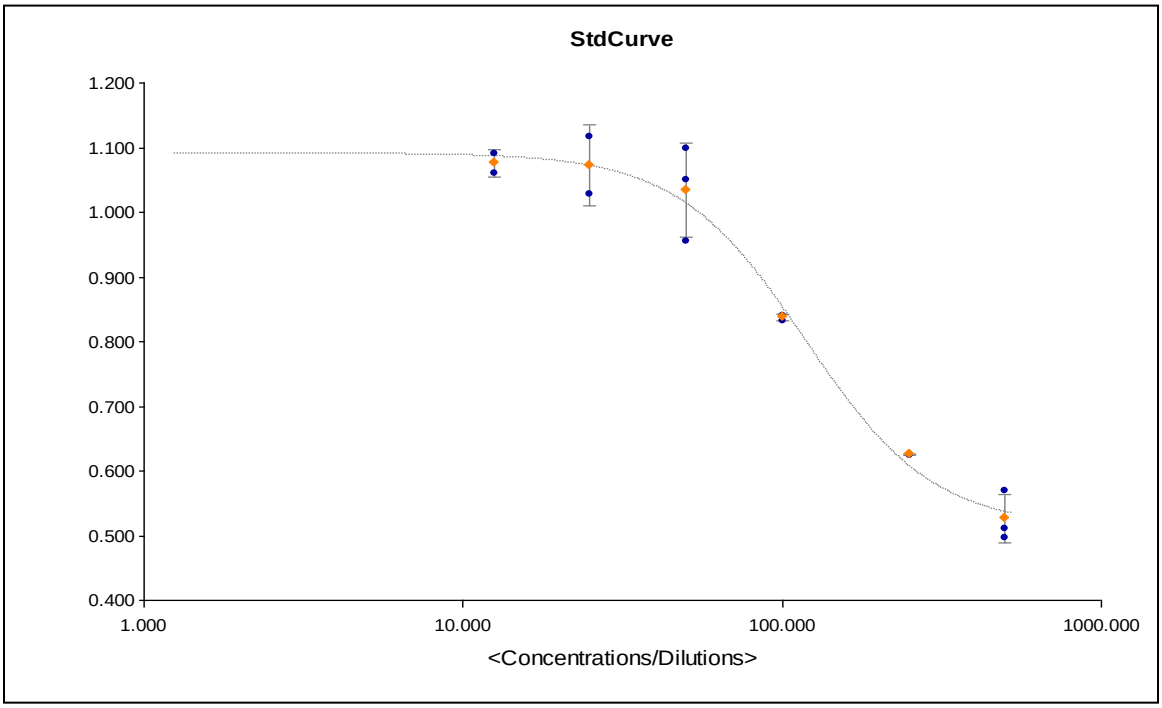
Sample duplicates had varying degrees of variability. Most duplicates had coefficients of variability below 0.1, but there was variability as high as 0.60-0.64 in three samples. Coefficients of variability across subjects were high, 57.9%.

Table 6. ELISA Measured Pre- and Post- Exercise Oxytocin Concentrations

	Pre-Max 1	Pre-Max 2	Pre-Max CV	Post-Max 1	Post-Max 2	Post-Max CV	Pre- SS 1	Pre- SS 2	Pre- SS CV	Post- SS 1	Post- SS 2	Post-SS CV
1	ND	ND		ND	ND		xxx	xxx		xxx	xxx	
2	ND	ND		808.29	671.51	0.13	ND	ND		ND	ND	
3	ND	ND		ND	ND		174.52	136.13	0.17	191.70	133.84	0.25
4	ND	ND		211.98	209.38	0.01	375.78	248.69	0.29	ND	ND	
6	ND	ND		ND	ND		ND	ND		xxx	xxx	
7	866.84	774.40	0.08	446.58	424.23	0.04	320.25	279.94	0.09	ND	ND	
8	ND	ND		ND	ND		xxx	xxx		511.30	623.51	0.14
11	ND	ND		ND	ND		344.89	364.31	0.04	244.41	249.40	0.01
12	323.58	334.90	0.02	ND	ND		94.27	250.11	0.64	271.04	198.25	0.22
13	ND	ND		558.84	1390.05	0.6	ND	ND		ND	ND	
14	ND	ND		1275.58	1179.16	0.06	ND	ND		ND	ND	
15	494.17	565.16	0.09	ND	ND		ND	ND		ND	ND	

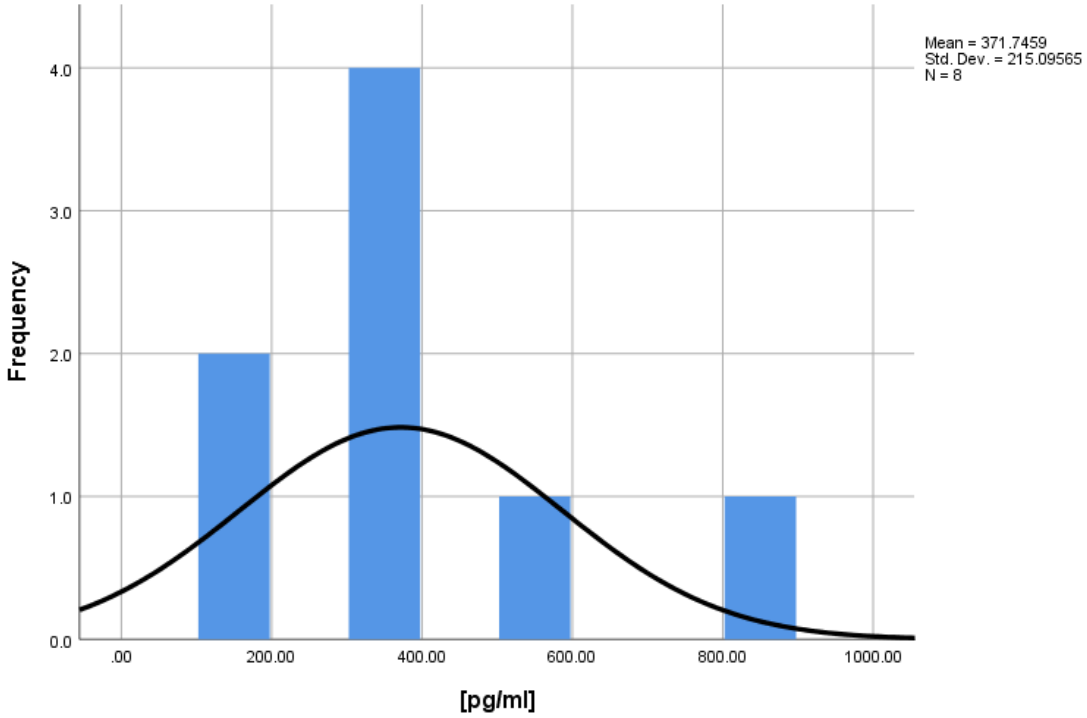
All values above are pg/ml. Values represented by "ND" were below the 22.9 pg/ml lower limit of detection and not detectable. Values represented by "xxx" signify concentrations higher than the standard curve allowed computation. Coefficient of variability (CV) is presented for each quantifiable duplicate. "Max" refers to graded maximal exercise test and "SS" refers to the 30 minute steady state exercise test. Coefficient of variability across subjects for all baseline measures was 57.9%.

Figure 1. ELISA Standard Curve



Y-axis values represent average optical density read at 450nm with the mean optical density of the non-specific binding wells subtracted out. X-axis concentration are pg/ml. r^2=0.996.

Figure 2. ELISA Measured Basal Plasma Oxytocin Concentration Without Values Below Detection Limit



Values above are based only on samples analyzed with the ELISA kit that had concentrations in the detectable and measurable range. Mean was 371.75 pg/ml, standard deviation 215.10 pg/ml.

Figure 3. ELISA Measured Basal Plasma Oxytocin Concentration with Values Below Detection Limit

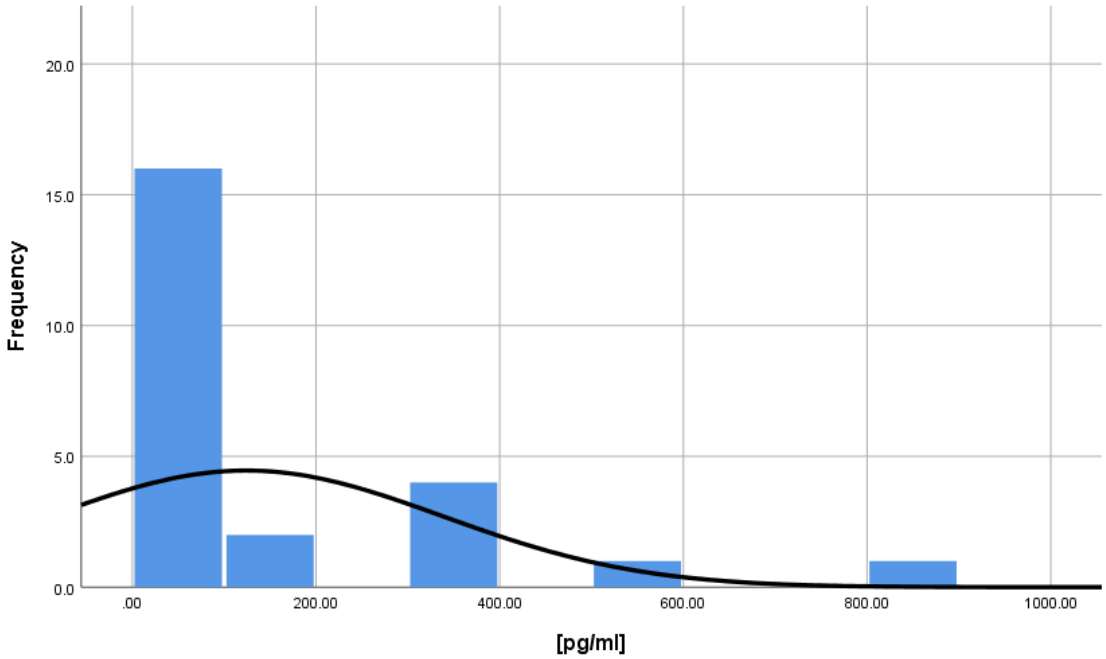


Figure 3 includes values below the detection limit of the ELISA kit. Values shown as 0.00 could not be quantified because they are less than 22.9 pg/ml. Mean concentration was 123.92 ± 214.77 pg/ml.

CHAPTER V

DISCUSSION

The ELISA methods described above was unable to accurately detect basal plasma oxytocin levels. The measured resting level using the extraction and ELISA kit was 371.75 ± 215.10 pg/ml. This value falls above the previously believed plasma basal levels for males and non-pregnant females of 1-10 pg/ml (McCullough et al. 2013) and below the reported levels of Brandtzaeg et al. (2016) of 600-800 pg/ml. ELISA methods rely on antibodies to bind specifically to the analyte of interest. The higher concentration values could be due to extra binding of the antibodies during the competitive binding assay possibly because of excessive proteins present in the samples or other contaminants. During the pilot experiments for the ELISA test, the first and second wash after plasma loading on the Sep-Pak column was collected. The first wash had too much degraded protein material in them to be analyzed. The second wash was analyzed in the competitive binding assay and had complete antibody binding (b%/b0 = 102%) thus demonstrating that no oxytocin was lost during this wash of the extraction.

The results of the ELISA test were puzzling. Roughly half (27 of 46) of the samples analyzed had values below the detectable level of the kit. The lower limit of detection for this ELISA kit was 22.9 pg/ml with a sensitivity of 17 pg/ml. It is unclear whether the inability to detect the oxytocin was related to the values being below the detection limit. This would be expected if the previously assumed 1-10 pg/ml basal

plasma oxytocin concentration in humans was correct or if the extraction technique utilized lost a significant amount of oxytocin. It should be noted that 800 µl of plasma was utilized in the purification process to enhance the amount by 8-fold. Thus if the plasma samples had 5 pg/ml, it was hoped that the amount would fall above the 22.9 pg/ml limit of this assay.

Based on the increase in subjects' heart rate during both exercise tests, the assumption can be made that the subjects experienced a normal exercise response. However, these two exercise stimuli failed to stimulate any detectable changes in plasma oxytocin. While it may appear exercise does not stimulate a change, it is possible that there is a dose dependent response with exercise. There may be a threshold of exercise intensity or duration required to produce a measurable difference in plasma oxytocin. For instance, Hew-Butler et al. 2008 observed a significant increase to plasma oxytocin following completion of a long duration ultra-marathon. Earlier, it was presented that this change was most likely due to changes in subject hydration, but it is possible the extended duration of the exercise stimuli also played a role in hormone shift. In contrast, the present study controlled for hydration state and this might have influenced the OXT response.

There were no apparent differences in oxytocin concentrations across genders. Both subjects with the highest baseline oxytocin concentrations (subject one and eight) were both males. Future research on human oxytocin measurements may benefit from assessing subject socio-emotional health prior to assessment. Some of the variation across subjects may have been due to differences in mental health.

Despite the accuracy and sensitivity reported by the referenced studies using paired high-performance liquid chromatography and the mass spectrometer, the present study failed to replicate these results. Based on the mass spectrometer calibration the lower limit of quantification was around 200 pg/ml. This is 200 times higher than the reported LLOQ of Zhang et al 2011 (1 pg/ml). Brantzaeg et al. 2016 reported linearity between 5-2000 pg/ml (r^2=0.99). It is possible the present study lost much of the oxytocin during the extraction if the bindings were not properly broken. The oxytocin levels in plasma, projected to be around 1-10 pg/ml, were extremely low and hard to detect with the mass spectrometer. Spiking all samples with 100 pg was required elevate them to the sensitive range of the equipment. However, a spike this large does present issues with data validity. Specifically, the spike of this size may reduce the ability to determine the initial plasma concentration. In addition, this amount of spike may mask small fluctuations in OXT that might occur. Even with this large spike, the inability to detect the oxytocin was baffling.

In future research these samples will hopefully be used and analyzed again via UPLC-MS to see if the values are consistant. This will help determine if the values obtained during the present study are accurate or are a result of contamination or artifacts of the analysis. If the results of this analysis show no oxytocin, the next step would be to develop a better extraction process using spiked samples.

The only divergence between the methods used for the UPLC in the current study and the methods used by Brandtzaeg et al. 2016, was the omittance of a solid phase extraction (SPE). Brandtzaeg used a SPE column on line to clean samples as well as

enrich and further concentrate them. This additional concentration of the plasma may be critical in accurate measurement of oxytocin. As stated earlier, the difficulty in detecting plasma oxytocin is in part due to the strong protein binding present in human plasma, as well as the staggeringly low concentrations seen in the plasma. A greater amount of plasma used in the extraction process might be necessary. While Brandtzaeg et al. (2016) used 100 µl of plasma during analysis, Zhang et al. (2011) used 1.4 ml of plasma and concentrated each sample prior to analysis. This process would alleviate the difficulties the mass spectrometer experiences when trying to detect oxytocin levels in the 1-10 pg/ml range. In the present study, 800 µl of plasma was utilized in the Eliza purification process to try to get samples into the detectable range. In the present study, the equipment used did not have the capabilities to add the additional SPE column on the UPLC system. Instead, cleaning the samples was accomplished by sample injection through a 0.22-micron filter after being centrifuged but before being put in the speed vac. Any protein that was disturbed during pipetting was removed by using this filter step. Increasing the amount of plasma used to increase absolute amount of oxytocin could have been utilized as well. Zhang et al. 2011 used 1.4 ml of human plasma combined with SPE before analysis via HPLC-MS. In the present study 800µl of plasma was utilized which was 4-fold higher than the amount suggested by Arbor Assays to further concentrate the samples to obtain greater amounts of OXT.

When comparing the two methods used to determine plasma oxytocin in this experiment, it is clear that the two are measuring different substrates. The assumed issue with the MS was the lack of detection below 200 pg/ml. However, those same samples

analyzed by the ELISA kit showed values mostly over that 200 pg/ml detection limit. If the ELISA measurements were accurate and specific, then the MS should have had no problem quantifying oxytocin concentration. The greater selectivity of the UPLC-MS combination used lends itself to two theories explaining the data: 1) the mass spectrometer is correctly attempting to measure only oxytocin, but the level in human plasma is too low for detection in the picogram for the UPLC-MS to detect, and 2) the extraction technique used prior to the UPLC-MS was too aggressive, and most if not all of the oxytocin was lost during the process. Regardless of which of these scenarios is correct, it is abundantly clear that this delicate process needs more research to decipher suitable extraction techniques and more in-depth sensitive and selective measures. In addition, the extraction used for the ELISA was Arbor Assays generic protein extraction. It appears oxytocin is a peptide hormone that might require a specialized protein extraction.

Based on the data measured from the ELISA, moderate intensity steady state and graded maximal exercises both fail to stimulate a sufficient stress response to upregulate plasma oxytocin secretion into the sensitivity range for this procedure. Although peripheral OXT may not have increased, central OXT may have increased. While social and mental stress tests have demonstrated the ability to induce these plasma hormone changes, physical stress may not (Jong et al. 2016). The details of the oxytocin response to stress is still somewhat a mystery and needs further research to determine what factors influence secretion centrally and in the peripheral.

Administration of oxytocin either nasally or intravenous has been proposed as a treatment for some anxiety disorders (Naja et al. 2017). Testing of the effectiveness of these drugs commonly uses different types of stress tests and measured oxytocin response to determine effectiveness of the treatment. While exercise tests are easily controllable and can be tailored specifically to each subject, it may be a poor stimulus of the stress response associated with plasma oxytocin change. Based on the data presented, researchers may want to avoid exercise tests in lieu of social and cognitive tests, such as the trier social stress test.

Physical activity had been shown to stimulate increased secretion of oxytocin in animals. Lang et al. 1983 used forced swimming as a stress stimulus and observed spikes in rat plasma oxytocin. While this response was seen in animals, it is less clear in humans. This may be due to differences in underlying mechanism, or it could be due to the sensitivity of the assays used. According it Brandtzaeg et al. 2016, rat plasma oxytocin is in much higher concentration than in humans. With a proper extraction technique, the next biggest issue surrounding oxytocin measurement is developing a repeatable sensitive measure that can detect the low amounts of OXT seen in human plasma.

While seemingly good methods exist for detecting plasma oxytocin in humans, many are very expensive, not sensitive enough, and are not reproducible. Future research should focus on creating reliable methods to measure this stress hormone. A better understanding of oxytocin could become key in the detection and treatment of a variety of anxiety and mental disorders. Learning oxytocin's underlying mechanism of actions

and further researching its role as a stress hormone may propel future studies on social and cognitive development, as well as identify risk factors for development of cognitive disorders

CHAPTER VI

CONCLUSION

Analysis of plasma oxytocin in humans requires the most precise instrumentation. The strong binding with proteins in the plasma must be broken prior to analysis which is troublesome for many methods. Resting oxytocin levels in humans is believed to be about 1-10 pg/ml, but has been recently reported to be closer to 600-800 pg/ml. The supposed low concentration previously believed could be due to improper extraction techniques being used or errors in the quantification process. However, the low concentration theory has more research supporting it. This low level adds further difficulty to the detection process. Using a thorough extraction process followed by high performance liquid chromatography and mass spectrometry has been reported to have the highest sensitivity for oxytocin measurement. Despite the best attempts at replicating the results of previous LC-MS studies, the present study failed to detect plasma oxytocin with mass spectrometry. While other studies have reported lower limits of detection around 1 pg/ml, the present study failed to have any detection below 200 pg/ml.

Quantification of plasma oxytocin was compared with that of a commercial ELISA kit. This kit had large coefficients of variability across subjects and thus, a normal basal level could not be confirmed. Issues surrounding the two methods used to extract the proteins seems to be a possible issue. While the UPLC-MS methods utilized a complex reduction, alkylation, and protein precipitation process, the ELISA extraction

methods relied heavily on separation through C18 Sep-Pak columns. It is suggested that these extractions procedures be investigated in the future.

Oxytocin changes in response to exercise in humans have been debated. This relationship is seen in rats and other animals but has mixed results in humans. The present study measured oxytocin before and after two exercise stimuli. A graded maximal exercise test and a moderate intensity thirty-minute steady state exercise were utilized for comparison. Our methods detected no difference in plasma oxytocin as a result of either of the exercise stimuli. It is unclear whether the lack of significant difference between exercise stimuli is due to a lack of physiological response, or due to the poor sensitivity of the methods used.

The data gathered in the present study support the idea that basal oxytocin concentrations normally are below 20 pg/ml. Unfortunately, due to the methods used, there is no direct data supporting this idea, but the lack of ability for the mass spectrometer to detect oxytocin levels suggest that resting levels must be below the detection limit of 200 pg/ml. The ELISA also had difficulties detecting oxytocin in many of the samples. This is hypothesized as having to do with the higher lower limit of detection reported by the kit manufacturer, 22.9 pg/ml.

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APPENDIX A

STANDARD OPERATION PROCEDURE FOR TOTAL OXT SAMPLE PREPARATION PRIOR TO LC-MS ANALYSIS

1. Place plasma samples, Tris-HCl, Internal Standard (IS), dithiothreitol (DTT) and iodoacetamide (IAM) on the lab bench. Wait until solutions reach room temperature so they do not absorb humidity from the air.
2. Add 100 µL plasma to a 1.5 mL eppendorf vial.
3. Add 200 µL Tris-HCl buffer (pH 8) to all vials.
4. Add 10 µL 10 ng/mL IS to all vials.
5. Vortex all solution at max speed for 10 seconds.
6. Add 5 µL 0.5 M DTT to all vials.
7. Place in oven at 37°C for 45 min.
8. Cool down to room temperature (approx. 10 min).
9. Add 15 µL 0.5 M IAM to all vials.
10. Place in darkness at room temperature for 20 min.
11. Perform protein precipitation (PPT) by adding 700 µL of 80% LC-MS grade acetonitrile (ACN) (cold). The water in the 80% ACN PPT solution will be solid ice in the freezer (-20°C). Therefore, remove the PPT solution from the freezer approx. 10 min before PPT is performed so that the ice melts.
12. Vortex all solution at max speed for 10 seconds.
13. Place the vials in the centrifuge and spin at approx. 12 000 rcf for 20 min.
14. Remove 900 µL of the supernatant over to new 1.5 mL Eppendorf vials
15. Perform speed-vac until dryness. Medium heat is advised (30-40°C).
16. Resolve the dried sample in 100 µL 0.1% FA.
17. Vortex approx. 20 seconds per sample to be sure that everything is resolved.
18. Centrifuge the resolved samples at approx. 12 000 rcf for 5 min.
19. Transfer supernatant to auto sampler vials.

APPENDIX B

PEPTIDE/PROTEIN EXTRACTION PROTOCOL ARBOR ASSAYS

1. Mix an equal amount of binding buffer (BB) to the plasma and vortex
2. Centrifuge at 6,000 x g to 17,000 x g for 20 minutes at 4°C. Collect supernatant
3. Equilibrate a C18 column by washing with 1 ml elution buffer (EB) followed by 3 x 3 ml BB.
4. Load the plasma/BB solution from step 1 onto the washed C18 column
5. Slowly wash the column with BB (3 ml, twice) and discard the wash.
6. Elute the peptide slowly with the elution buffer (3 ml, once) and collect eluant in a polypropylene tube.
7. Remove acetonitrile solvent in a centrifugal concentrator. Freeze-dry results solution to dryness
8. Dissolve the residue in a suitable volume of assay buffer provided with the kit.

APPENDIX C

ELISA PROTOCOL ARBOR ASSAY CATALOG NUMBER K048-H1

1. Pipet 100 µL of samples or standards into wells in the plate.
2. Pipet 100 µL of Assay Buffer into the maximum binding (B0 or Zero standard) wells.
3. Pipet 125 µL of Assay Buffer into the non-specific binding (NSB) wells.
4. Add 25 µL of the DetectX® Oxytocin Conjugate to each well using a repeater pipet.
5. Add 25 µL of the DetectX® Oxytocin Antibody to each well, except the NSB wells, using a repeater pipet.
6. Shake the plate in a plate shaker at room temperature for 15 minutes to ensure adequate mixing of the reagents. Cover the plate with the plate sealer and store at 4°C for 16-18 hours.
7. The following day, remove the TMB Substrate from the refrigerator and allow to come to room temperature for at least 30 minutes. Addition of cold Substrate will cause depressed signal.
8. Aspirate the plate and wash each well 4 times with 300 µL wash buffer. Tap the plate dry on clean absorbent towels.
9. Add 100 µL of the TMB Substrate to each well, using a repeater pipet.
10. Incubate the plate at room temperature for 30 minutes without shaking.
11. Add 50 µL of the Stop Solution to each well, using a repeater or a multichannel pipet.
12. Read the optical density generated from each well in a plate reader capable of reading at 450 nm.