To Evaluate the Function of the Oxytocin Receptor in the Context of Ovarian Cancer Cell Microenvironment to Determine if Oxytocin can Induce an Anti-Inflammatory Response

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Benjamin I. Schachner

TO EVALUATE THE FUNCTION OF THE OXYTOCIN RECEPTOR IN THE CONTEXT OF OVARIAN CANCER CELL MICROENVIRONEMENT TO DETERMINE IF OXYTOCIN CAN INDUCE AN ANTI-INFLAMMATORY RESPONSE

by

BENJAMIN I. SCHACHNER

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Thesis Chair: Dr. Mohtashem Samsam

ABSTRACT

The treatment of most cancers can still be considered inadequate despite the steady progress being made. A prime example of this issue is with epithelial ovarian cancers; this disease presents a significant issue, with a 5-year survival rate of 46% and a survival rate of 28% in patients that develop metastatic disease. Since ovarian cancer has such a high mortality rate, effective treatment modalities are necessary to prolong the quality of life after diagnosis. Psychosocial stress is related to the progression, proliferation, and migration in cancer patients, but the mechanisms of this relationship are not fully understood. The present *in vitro* study investigated the ability of oxytocin, a neuropeptide associated with social support, to attenuate the stress response. Catecholamines, a subclass of stress hormones, were used to simulate the stress induced inflammation process in ovarian cancer cells. To evaluate oxytocin's capacity to attenuate the stress response, the ovarian cancer cell lines SKOV3, HEYA8, OVCAR8, and OV432 were separately treated with the presence or absence of catecholamines with the addition of oxytocin. Protein expression of the oxytocin receptor was investigated using a western blot protocol. Oxytocin receptor, oxytocin, and IL-6 mRNA expression was evaluated by quantitative PCR. Treatment with Oxytocin attenuated the inflammatory response resulting from catecholamine treatment. The oxytocin receptor gene and protein were present in each cell line, suggesting that oxytocin has an anti-inflammatory role in the tumor microenvironment in ovarian cancer patients. These results provide a mechanism by which social support, working through the release of oxytocin, promotes an anti-inflammatory process in ovarian cancer patients. This study may shed light into new pharmacological approaches for the treatment of ovarian cancer.

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PURPOSE AND SIGNIFICANCE

This research aims to characterize the function of oxytocin/ oxytocin receptor (OXT/OXTR) in human ovarian cancer cells. Given the presence of OXT in the ascites fluid of ovarian cancer patients, and the inverse correlation found between OXT levels and inflammation makers (as reported to us from the Lutgendorf lab), we will evaluate a possible cellular mechanism in ovarian cancer cells. The increased levels of oxytocin found may serve to modulate the inherent inflammatory state of the cancer cells to potentially modulate tumor growth and progression. We will evaluate if OXT/OXTR in ovarian cells is present to modulate the inflammatory state. We believe OXT/OXTR expression will be increased during inflammation and that oxytocin may act as a stress buffer in this situation. By investigating the correlation between any changes observed in these cells with oxytocin treatment it may open new doors for ovarian cancer treatment. By understanding the mechanism potentially at work we may be able to advance a pharmacological approach to both pre-clinical and clinical models.

CHAPTER ONE: INTRODUCTION

Definition of Stress and Relevant Pathways

When receiving the diagnosis of cancer, and life thereafter, the patient can find many of the aspects of the disease to be stressful, such as the physical, emotional and social effects. This stress disrupts the body's homeostasis and in this context described as "the state of being threatened, or perceived as being threatened" [1, 2]. This disruption of homeostasis is associated with activation of the body's two major stress systems, the sympathetic nervous system and the hypothalamic- pituitary-adrenal (HPA) axis[3]. The physiological reaction to stress includes arousal of the HPA axis above the normal level, which produces the secretion of cortisol and stimulation of the sympathetic nervous system (SNS) which promotes the secretion of catecholamines[2, 4]. Noradrenergic pathways from the SNS have been linked with the growth and progression of ovarian cancer. Further, bio-behavioral factors have been linked to norepinephrine levels in the tumor microenvironment; concentrations in ascites are unknown, although spillover from perivascular neuro-muscular junctions could be a possible pathway in ovarian cancer patients[5]. The idea that psychosocial elements influence the progression and growth of cancer has been an accepted theory for many generations. To validate this hypothesis epidemiological and clinical studies have illustrated that there is a link between chronic stress, being in a state of depression and being socially isolated with cancer growth [3, 6-9]. Current studies of the microenvironment of carcinomas have distinguished particular signaling pathways that effect cancer progression and metastasis from stress molecules (catecholamines). It is relevant to note that catecholamines have been shown to increase the secretion of inflammatory molecules from cancer cells, including ovarian cancer cells[2, 3].

Molecules of Interest Released During "Stress"

Stress can be acute or chronic in nature[10]. Concerning the psychological stress of cancer diagnosis and treatment, chronic stress is of importance to cancer patients and this present study. When an individual experiences chronic and continuous stress, the body's system stays in a condition of elevated work. This is harmful in regards to the body's management of stress response systems, as well as other internal systems [3]. Individuals with chronic stress are known to have raised levels of the catecholamines norepinephrine (NE) and epinephrine (E) [11]. Of note, studies on this phenomena have shown that chronic stress stimulates tumor growth, angiogenesis, and metastasis [3, 12, 13].

Previously, the pro-inflammatory/pro-angiogenic cytokine interleukin-6 (IL-6) has been associated with chronic stress[2]. Specifically, those who have documented chronic stress have been shown to have above average levels of circulating IL-6 [14]. Further, behavioral components have been linked with both the levels of circulating and ascites IL-6 in ovarian cancer patients [2, 15]. The catecholamine E has been demonstrated to increase IL-6 expression in adipose tissue [16], and NE treatment of myocytes increases IL-6 expression [17]. Most importantly, catecholamine treatment lead to elevated expression of IL-6 in ovarian cancer cells[2]. The effect of NE and E on IL-6 expression was inhibited by using a β-adrenergic receptor antagonist, however an α-adrenergic receptor antagonist was not able to elicit the same effect [2]. This is evidence that relevant stress hormones activate β-adrenergic receptors on ovarian cancer cells[2, 18]. This receptor pathway increases the kinase activity of Src, this increases transcription of the IL-6 promoter and as a result increased production of IL-6

protein[2]. To clarify, Src tyrosine kinase is activated through phosphorylation via the cAMP/PKA signal cascade, which is required for increased IL-6 expression[2].

Effects of Elevated IL-6 in the Microenvironment Due to Stress

IL-6 is elevated in chronically stressed ovarian cancer patients, [14]. However, the mechanism that regulates IL-6 secretion, as well as its influence in the tumor microenvironment remain to be determined. High levels of IL-6 are often observed in the serum of ovarian cancer patients[2]. Further, this finding is correlated with a poor outcomes and increased tumor size [19, 20]. Ovarian cancer cells express β-adrenergic receptors, and stimulating these receptors with NE produces increased expression of vascular endothelial growth factor (VEGF), an important pro-angiogenic factor [12, 21]. Angiogenesis is the formation of new blood vessels, which involves the migration, and growth of endothelial cells. This process is associated with VEGF, and is required for tumor growth and ascites formation[2]. This is regulated by the presence or absence of pro-angiogenic and anti-angiogenic molecules [13, 22].

IL-6 released by ovarian cancer cells further facilitates tumor cell growth, and metastasis, as well as chemotherapy resistance [2, 23-25]. The receptor for IL-6 has been shown to be present on endothelial cells in the ovary[26]. Treatment of ovary endothelial cells with IL-6 *in-vitro* results in increased cell migration, which implicates its role in as a pro-angiogenic factor *in-vivo* [26].

Social Support has been Shown to Act as a Protective Factor

It has been suggested that social support can act as a buffer, or protective factor for the progression and metastasis of cancer, and could be associated with cancer survival advantage.

Researchers deem social support important when dealing with a crisis, including psychological adjustment to cancer [27-30]. In a study with breast and gynecological cancer survivors it was observed that social support was a strong determinant on the quality of life[31]. Further, it was seen that social support was positively correlated with health-related quality of life in breast cancer patients[32]. Social isolation in similar circumstances yielded important findings as well. Social isolation, meaning the lack of friends, nuclear and extended family, after breast cancer diagnosis was correlated to poorer outcomes[33]. It would appear that social support acts as buffer to progression of disease. The importance of social support was illustrated by research showing that there is a correlation between social support and quality of life in both gynecological and breast cancer survivors. [33-35]. Furthermore, social support is instrumental in coping with psychological issues that stem from the cancer diagnosis, such as anxiety and depression. These psychological issues are seen often in cancer patients and can contribute to physiological stress[6]. These results taken together suggest that social support may be a protective factor in cancer progression and metastasis, pointing to the importance of underlying physiological mechanisms linking social support to the tumor microenvironment[8, 9, 30, 36].

How Oxytocin is Released and Circulated in the Body

Oxytocin (OXT) has been shown to be active in the neurobiological mechanism that promotes social attachment and bonding. OXT is known for its traditional roles in parturition and lactation [37]. OXT is synthesized in the paraventricular nucleus (PVN) and supraoptic nucleus of the hypothalamus[38]. Magnocellular oxytoningeric neurons in PVN send their terminals to the posterior pituitary where they release oxytocin into portal circulation [38, 39]. Parvocellular

oxytonergic neurons in the PVN send axons to multiple brain and spinal cord areas important for autonomic nervous system control and processing of painful stimuli[40]. These projections include other hypothalamic nuclei, the median eminence, the amygdala, hippocampus, locus coerulues, striatum, raphe nuclei, the dorsal motor nucleus of the vagus nerve, and the nucleus tractus solitarius [40]. Recent studies have demonstrated that OXT plays a role in social behaviors and the stress response [41, 42]. Specifically, oxytocin is involved in affiliative behaviors such as maternal care, sexual behavior, monogamous social bond formation, and social recognition [41]. The release of OXT to the bloodstream and relative projections from affiliative behaviors suggests multiple pathways OXT may be involved in, in both the CNS and local environments[43-45].

While the exact role of OXT in affiliative social interactions remain under investigation, it has been hypothesized that OXT may counter SNS activation under conditions of social recognition or affiliation, thereby buffering the stress response and facilitating affiliative behaviors [46].

Why Oxytocin is the Molecule of Interest

The peptide oxytocin (OXT) has been suggested to be active in a mechanism underlying the positive results associated with social support that influence cancer progression[47-49]. OXT is known for its use to induce labor by targeting the uterus[37]. Ovarian tissues and other parts of the female reproductive organs have been shown to express oxytocin receptors (OTR)[37]. Thus, OXT has the potential to have a role in regulation of the ovarian tumor microenvironment.

The oxytocin receptor (OTR) is a 389 amino acid polypeptide that has seven transmembrane domains. This receptor is a G protein coupled receptor [50]. The oxytocin receptor is present in two different lengths, one size in the breast and a larger size in the ovaries, endometrium and myometrium [50]. One of the known roles of OXT, as discussed above, is its role in milk ejection reflex. The stimulation of the infant suckling on the nipple generates a pathway that starts at the nipple; the impulse is then transmitted to the spinal cord, and from there to the secretory oxytonergic neurons in the hypothalamus[37]. These neurons have high frequency burst activity, which leads to a large release of OXT into the blood stream, which then migrates to the breast[37].

The Link Between OXT and Cancer Cells

OXT receptors are present in numerous breast cancer and other breast related cell lines. The activation of the OXT system might be the reason breast feeding is linked to a lower occurrence rate of breast cancer among mothers that breastfed compared to those who did not; specifically breast cancer after menopause [51]. OXT receptors in human breast tumors and breast cell lines reacted to OXT by a buildup of calcium within the cell, activation of ERK-2 phosphorylation, and PGE2synthesis[52].

It is believed that the ovary is a site of OXT production for local use [53]. This local oxytocin is believed to have a role in fertilization and maturation of the embryo in humans [54]. A hypothesis of this present study is that local oxytocin is released by ovarian cancer cells, which has the potential to be active in an anti-inflammatory pathway, as well as influencing the synthesis of PGE2.

In the recent study by the Lutengorf lab and colleagues, there was a significant positive relationship between social attachment and survival advantage for patients with ovarian cancer[9]. Thus, although the clinical relevance has been observed, the mechanisms linking social attachment and survival are not fully understood. The present study seeks to define these mechanisms *in vitro* better understand the association between social support and ovarian cancer outcomes.

Oxytocin has the Ability to Inhibit Cancer Progression and has Systemic Anti-Inflammatory Effects

Affiliative social behavior, through the release of OXT, has been observed to inhibit cancer progression in animal models. Specifically, it inhibits proliferation, migration, and invasion of cancer cells as well as increased expression of E-Cadherin when mice inoculated with SCOV3 cells were treated with OXT [47]. Further, OXT decreased intraperitoneal dissemination of the tumor. Intraperitoneal dissemination is a common pathway of tumor progression that results in the spread of the tumor and the accumulation of ascites fluid. The increased expression of E-Cadherin has an important function in cell adhesion, the loss of its function is linked to greater tumor metastasis[55].

To examine mechanisms of this phenomenon *in-vivo* the OXT effect on the CNS should be examined. It is possible that OXT reduces CNS activity while increasing peripheral nervous system (PNS) activity, though the reason is not well understood [45]. When the parasympathetic nervous system is activated, there is reduced inflammation and oxidative stress in models of acute tissue injury and inflammation [56]. Mechanistic explanations for the potential stress

buffering role of OXT come from neurophysiological research suggesting that OXT can enter the pituitary portal system through the median eminence to directly affect secretion of anterior pituitary hormones including adrenocorticotropic hormone (ACTH), growth hormone, and prolactin [57]. Studies have also found species-specific effects indicating that OXT causes short term increases in ACTH and corticosterone secretion in rats, but inhibits these hormones in humans and nonhuman primates, which does not offer much of an explanation for the broad anti-inflammatory theory[58].Other lines of research indicate that long term OXT treatment also lowers corticosterone concentration, and increases cholecystokinin levels. This suggests that OXT may be decreasing SNS activity and increasing vagal activity, implying a general shift from sympathetic to parasympathetic activation [45]. Other research further demonstrates that OXT administration causes a shift toward vegetative function, including increased energy storage. For example, Intracerebroventricular injections of OXT can promote the release of the gastrointestinal hormones, insulin and glucagon, through activation of vagal efferents that innervate the GI tract and pancreas [45, 59]. This research supports the hypothesis that OXT counters the SNS, but the systemic actions may not be a full explanation for phenomena observed, such as wound healing and decreases of inflammation across a variety of disease states.

Oxytocin can Influence the Cell Microenvironment

In addition to its CNS and systemic actions, OXT is produced locally in a variety of peripheral tissues in both sexes, suggesting that this peptide may be involved in the basic physiological functions of several organs[37]. Recent research has revealed a novel effect of

OXT on various cancer cell lines [48, 60]. In this situation, OXT functions as a growth regulator by activating the oxytocin receptor (OTR). When the OTR is stimulated two different pathways can be activated. There can be an increase or inhibition of cell proliferation because of the OTRs ability to activate either the Gi or Gq proteins[61]. The Gq pathway triggers the hydrolysis of PIP2 by PLC, this increases the cystolic calcium level[61]. The increase in calcium increases the proliferation and growth of some cancer cells. The Gi pathway inhibits PKA-induced tumor growth and increases the expression of $p21^{Waf1}$ via the ERK pathway. This pathway inhibits cell growth[61]. *In vitro*, OXT was able to inhibit the growth and multiplication of cancer cells, this was observed in cancerous cells of both epithelial and bone origins. These cells all expressed OTR [48, 62]. The ability of OXT to inhibit proliferation was observed in mouse and rat ovarian carcinomas *in vivo* [47]. This is an important finding as this study aims to define a pathway that could explain this result. These results are not always the case, in other cancer cell lines treatment with OXT increased growth [63]. The signal pathway for growth inhibition or stimulation appears dependent on OXT/OTR binding to Gi or Gq. OXT can inhibit growth through the activation of the cAMP-PKA pathway through binding and activating the Gi pathway. The growth can occur through the activation of the Gq protein which when activated increases intracellular calcium and tyrosine phosphorylation. This Gq activation was the known and accepted function of OXT for many years [63, 64]. The unexpected role of OXT in managing cell growth open new perspectives on the role of the OXT/OTR system in various cancer cells, noting that many cancer cells of different origins express OTR. This suggests the potential importance of peripheral OXT in local regulation of tumor growth and metastasis.

These considerations lead to the hypothesis that positive social environment may influence the progression of ovarian cancer at a local level.

OTR expression has been observed in breast cancer cells (as discussed above), endometrial cancer cells, glial tumor cells, immune cells, and in neuroblastoma cells. In these tumors OXT was able to inhibit their growth [48, 65-68]. It is of importance to note that there was an increase in cell growth after stimulating the OTR in small cell carcinoma of lung cell lines and choriocarcinoma cell lines, which is probably mediated through the Gq pathway [63, 69]. Speculation of why the OTR-mediated effect varies in many types of cancer, though the inhibition of cancer proliferation, growth, and metastasis by OXT presents a possible pharmacological pathway.

Previous data has shown that in individuals with ovarian cancer the influence of psychosocial well-being was correlated with positive outcomes[9]. OXT has also been demonstrated to inhibit inflammatory molecules of interest such as IL-6 and VEGF, across a wide range of medical diseases, including ovarian cancer[15, 70]. Preliminary data from Lutgendorf and colleagues has shown that higher levels of OXT in ascites are associated with higher quality of life, psychological well-being, and positive affect. Defining the biopsychological mechanism, which explains why social support has been shown to increase quality and length of life in ovarian cancer patients, is the overarching goal of this study.

While pathway mechanisms and possibilities remain controversial[61], research investigating the activation of oxytocinergic systems through administration of OXT has provided evidence for the existence of an OXT pathway that could convey protection against the progression of ovarian cancer[9, 47]. Thus, one possible mechanism mediating the beneficial

effect of positive social environment on ovarian cancer progression is the activation of a local oxytocinergic system that modulates inflammatory and oxidative stress pathways.

The known Oxytocin Anti-Inflammatory and Anti-oxidant Pathways

Although there is research supporting the notion that OXT counters SNS activity, it does not explain the anti-inflammatory pathways observed in many *in-vitro* and *in-vivo* models. Treatment with OXT was able to enhance wound healing, possibly through increased insulin-like growth factor production [71]. In the carrageenan hind paw inflammation model, OXT was found to increase nociceptive thresholds and decrease both edema and tissue myeloperoxidase activity [72]. The level of Myeloperoxidase (MPO) activity in tissue is a marker for neutrophil recruitment into the intravascular space, which increases inflammation [73]. The release of reactive oxygen species by MPO activity from infiltrating neutrophils contributes to many inflammatory conditions through damage to cell membranes, lipid peroxidation, and activation and recruitment of macrophages [74]. OXT administration has also been shown to decrease MPO activity in acetic acid induced colitis in rats [75]. In the same study, peripheral OXT administration inhibited circulating TNF-α. TNF-α is responsible for increased neutrophil infiltration during conditions of acute inflammation through upregulation of the chotactic factor, MCP-1, on circulating leukocytes and endothelial cells [76]. This suggests that OXT decreases TNF-α levels, which could be responsible for the observed decreases in tissues MPO activity.

Findings from our laboratory suggest that OXT receptors exist on monocytes, macrophages, endothelial cells, and smooth muscle cells, and that OXT inhibits TNF-α stimulated NAD(P)H-oxidase activation in these cells *in vitro*[77]. However, expression of

OXT/OTR as well as if OXT exerts potent inhibitory effects on proangiogenic and pro-inflammatory molecules released by ovarian cancer cells such as IL-6, VEGF, and TNF-α remains unknown.

The mechanism responsible for the inhibitory effects of OXT requires clarification. Some experimental findings have suggested that the anti-inflammatory and anti-oxidant effects of OXT are due to CNS mechanisms, while others have excluded this possibility by demonstrating that administration of OXT has no such effects [78]. Administration of high doses of OXT can induce glucocorticoid release and some authors have suggested that the observed anti-inflammatory actions of OXT are mediated by increases in endogenous steroids [79]. However, others have shown similar effects at lower doses that were unlikely to impact glucocorticoid levels [72].

The present study evaluated the possibility that OXT acts directly on peripheral OTRs to dampen inflammatory and oxidative stress effects through purely local mechanisms. As mentioned above, support for this hypothesis comes from research suggesting OTR activation can inhibit IL-6 protein secretion in both THP-1 macrophages and endothelial cells [77]. OTR promoter region is co-localized with those of pro-inflammatory cytokines meaning that this peptide may influence transcription of inflammatory genes [80]. The most likely explanation for the observed anti-inflammatory and anti-oxidant effects of OXT *in-vivo* is the down regulation of pro inflammatory cytokines and reactive oxygen species production. One drawback of this hypothesis is that at high, but physiologically relevant doses, OXT has significant affinity for the vasopressin receptor [81]. The effects of vasopressin receptor activation can be just as important to those of OTR in researching the effects of OXT, depending on ovarian cancer gene expression

of vasopressin receptor. Further, dose response to OXT has not been studied in the ovarian cancer cell lines being used relative to IL-6 secretion.

As discussed above, when evaluating cancer prognosis it is believed that psychological factors play a role in cancer growth and progression[3]. The lack of social support and the feeling of hopelessness are believed to effect the growth and progression of cancer through the effect of glucocorticoids on cancer cells. Numerous studies have observed that cortisol treatment leads to an increase in cancer cell invasiveness, dependent on the cell lines [18].

OXT was able to reverse the effects of cortisol through the induction of autophagy in the SKOV3 cell line. This was confirmed through the noted increase of the Beclin-1 gene[49]. Of note, this study used a high concentration of OXT, a dose that is many folds higher than physiological levels. Since synthetic glucocorticoids can enhance cell survival in epithelial tumors, such as ovarian cancer tumors, it is evident that OXT has an important role in the tumor microenvironment.

Most importantly, to avoid nausea and vomiting post-surgery dexamethasone is given to patients with ovarian cancer. Multiple studies found that ovarian cancer patients treated with dexamethasone had an increase of pro-cancer cell survival genes at the tumor site [82-84]. Further, dexamethasone increased chemotherapy therapy resistance of ovarian carcinomas *in vivo*, and increased basal growth of the xenografts[85, 86]. It is possible that glucocorticoids interfere with chemotherapy effectiveness.

In this context, OXT may be effective through reversing the harmful effects of glucocorticoids on cancer cell survival [49]. This opens new perspectives on OXTs role and possible pharmacological use in ovarian cancer patients.

Ovarian Cancer

Ovarian cancer (OVC) can be split into two groups, epithelial ovarian cancer and non-epithelial ovarian cancer, the latter comprising approximately 10% of ovarian cancers. Approximately 240,000 people are diagnosed with ovarian cancer per year [87]. Although OVC is less common than other cancers, it is one of the most deadly cancer types because unspecific symptoms and the lack of biomarkers make it difficult to detect before metastasis and/or mortality. The 5 year survival rate of epithelial ovarian cancer is 46%, stage III (tumor has spread to the peritoneum outside of the pelvis) having the lowest long term survival rate at 35% [87]

There are 5 major subtypes of OVC: High-grade serous carcinomas (HGSOC), endometrioid carcinomas, ovarian clear cell carcinomas, mucinous carcinomas, and low grade serous ovarian carcinomas [88]. In order to evaluate the anti-inflammatory effect of oxytocin on ovarian cancer, four unique cell lines were used.

HGSOC is the most common ovarian cancer subtype, making up 70% of all epithelial ovarian cancers [89]. This tumor is highly proliferative which could characterize it as aggressive[90], and HGSOC tumors are known to be chemotherapy resistant [91].

In conclusion, the beneficial effects of social support for ovarian cancer patients may be mediated through an increase of OXT in the tumor microenvironment. This study will evaluate OXTs effect on pro-inflammatory biomarkers in ovarian cancer cells specifically. Further studies are necessary to test and develop the clinical use of OXT.

CHAPTER TWO: METHODS

Cell Culture

Cell lines used for this study were SCOV3/SKOV3, OVCA432, HEYA8, and OV432. The cell line SCOV3 is a human ovarian cancer cell line that has epithelial like morphology. The cell line was established in 1973 from the ascites of a 64-year-old Caucasian female with adenocarcinoma of the ovary [92]. SKOV3 is unique as its immune phenotype most closely resembles high grade serous, but also resembles clear cell (OCCC) histology. OCCC are similar to endomertrioid and they are limited to the ovary at diagnosis. [92]

The next cell line used was OVCA432. This is another high grade serous ovarian cancer cell line; there is variation in how these cells react, and is heterogenetic in nature[93]. OVCA432 cell lines are not well documented with varying reports on classifications. It is most reported to be an ovarian serous adenocarcinoma. It has been reported that OVCA432 secretes a modest amount of VEGF compared to other OVCA cell lines.[93]

HEYA8 cell line is a high-grade ovarian serous adenocarcinoma, which share lineage with the HEY ovarian cancer cell line. This cell line is a moderately differentiated papillary adenocarcinoma, and is considered one of the more aggressive cell lines based on survival time and growth rate in pre-clinical models[94, 95].

OVCAR8 is considered a high grade ovarian serous carcinoma. This cell line is documented to be similar to HEYA8, but is not as proliferative[95]. There is limited relevant information available on this cell line.

It should be noted, all four cell lines are widely used but there are significant misidentifications, and many duplicates out there[96]. This is of importance to this study, as results should be tested *in-vivo*.

OV432, SCOV3, OVCAR8, and HEYA8 cells will be maintained in a DMEM medium containing 10% fetal bovine serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and incubated at 37°C in humidified 95% air-5% CO_2 incubator. Cells were seeded at 10,000 cells/well in 24-well tissue culture plates or into 60- or 100-mm dishes at 1 x 10^6 or 5 x 10^6 cells/dish, respectively, and maintained on growth media until confluent. Cells will be incubated with OXT at physiologically relevant levels (10-1000 pM) and processed for downstream application and analysis.

PCR Experiments for Measuring IL-6 and OXTR mRNA Expression

The Rt-PCR experiment protocol follows the one set forth by the Mendez lab from previous studies [77].

At the end of the incubation, cells will be washed three times with Dulbecco's PBS. 350 μl of RLT Lysis Buffer (Qiagen RNeasy Kit, Qiagen, CA) will be added to each dish. Cells will be detached by gentle scrapping and will be collected in QIAshredder spin columns placed in 2 ml collection tubes. Columns will be spun at maximum speed for 2 minutes and samples frozen at -80°C until ready for RNA isolation. OXTR, OXT, IL-6, VEGF, or any other relevant mRNA expression will be evaluated by real time polymerase chain reaction (RT-PCR). Total RNA (optical density ratio of 260/280 nm, > 1.8) will be isolated using the RNeasy Kit (Qiagen, CA) and stored at -80°C. After treatment with DNAse I (Applied Biosystems), the reverse transcriptase reaction will be carried out by mixing 5–10 μg of total RNA in the presence of

random oligomers (3.2 μg), $MgCl_2$ (5 mM), deoxynucleotide mix (1 mM), Avian Myeloblastosis Virus (AMV) reverse transcriptase (≥20 U), and RNase inhibitor (50 U) and incubated at 25°C for 20 min, then at 42°C for 60 min, denatured at 99°C for 5 min and then cooled to 4°C for 5 min, resulting in cDNA synthesis. Quantitative gene expression of human OXTR, OXT, IL-6, VEGF, or other genes of interest by RT-PCR will be performed with the TaqMan gene expression assay (Applied Biosystems). Fifty nanograms of cDNA will be amplified with TaqMan Universal PCR Master Mix and reactions will be run using universal cycling conditions on an Applied Biosystems 7500 Real-Time PCR system. Samples will be analyzed in triplicate for accuracy. The ΔΔCT (threshold cycle) method will be used to analyze changes in gene expression in a given sample relative to another reference sample and expressed as the fold change in gene expression. 18*S* mRNA will be used as the endogenous mRNA control. A non-template control will be performed to ensure that there will be no amplification of genomic DNA.

IL-6 Assay

Cells will be incubated after treatment under relevant experimental conditions for 6 hours, and then cell-free supernatants will be assayed by enzyme-linked immunosorbent assay (ELISA) for IL-6 using commercially available reagents (BD Biosciences, San Diego, CA). As an indicator of inflammation, all IL-6 secretion concentrations will be normalized to cell protein concentration.

OXT Extraction

Ovarian cancer cell lines SKOV3, HEYA8, and OVCAR8 were grown to confluence and incubated for 16 hours, after which the media and cells were collected. Secretion of oxytocin was measured in culture media after solid phase extraction by ELISA (Arbor Assays, Ann Arbor MI) as previously described [43].

Statistical analyses.

Data were obtained from a minimum of three replicates from at least three separate experiments and are presented as means ± SE. Results were compared by paired independent *t*-tests or ANOVA (1- or 2-way) with post hoc Bonferroni correction. An α-level of 0.05 was required for statistical significance.

CHAPTER THREE: RESULTS

Oxytocin receptor expression

Expression of OXTR in four ovarian cancer cell lines was quantified by rt-PCR (Figure 1A). SCOV3, HEYA8, OVCAR8 all expressed detectable levels of OTR mRNA. Expression was highest in OVCAR8, followed by SCOV3, HEYA8, and OXTR was not detectable in the OV432 cells. OXTR protein expression was examined in these cells by immunoblotting (Figure 1B) and demonstrated two major immunoreactive bands with apparent molecular mass of 43 and 57 kDa This is consistent with the molecular weights of the native, unglycoslyated and the mature glycosylated forms of the receptor, respectively [50]. Relative expression of the OXTR protein, normalized to levels present in SCOV3 cells, demonstrated that HEYA8 cells exhibited the highest expression levels, followed by OVCAR8, SCOV3, then OV432 cells. The 57kDa band exhibited a twofold, and 1.5 fold increase of protein expression in HEYA8 cells and OVCAR8 cells compared to SCOV3, respectfully. In the 43kDa band, HEYA8 had an eightfold increase in protein expression compared to SCOV3, and OVCAR8 showed a fourfold increase in protein expression compared to SCOV3. Notably, the relative expression of OXTR mRNA and protein were not concordant in the different cells lines such that mRNA expression did not predict protein expression.

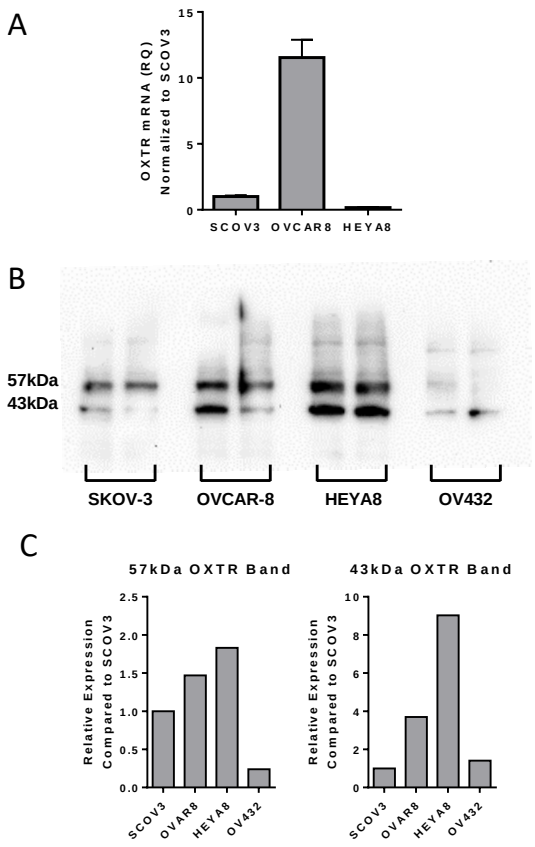

Figure 1: A: Expression of OXTR mRNA normalized to SCOV3. B: Expression of oxytocin receptor protein expression in human ovarian cancer cells SKOV3, OVCAR8, HEYA8, and OV432. C: Expression of the glycosylated and unglycosylated OXTR relative to SKOV3.

Effect of Norepinephrine on OXTR expression in human ovarian cancer cells

Norepinephrine (NE) is released from the sympathetic nervous system during stress, and research show that chronic stress and other psychological conditions that increase NE production are correlated with the spread and growth of cancer[18, 21, 97]. One consequence of norepinephrine activation of adrenergic receptors on ovarian cancer cells is upregulation and increased secretion of IL-6 (and VEGF) that can contribute to tumor proliferation and increased tumor vascularization [3]. To evaluate the effect of norepinephrine as an inflammatory stimulus on SCOV3, HEYA8, OVCAR8, and OV432 cells were treated with norepinephrine for 6 hours. Following treatment, cellular mRNA was isolated to evaluate gene expression of IL-6 as a marker of inflammation as well as effects on OXT and OXTR (Fig. 2). IL-6 mRNA expression increased significantly with norepinephrine treatment in SCOV3, HEYA8, and OVCAR8, which that confirms published results ($p<.05$) [2]. Previous data have revealed that OXTR is upregulated in macrophages during an inflammatory response [98], thus the inflammatory effects of norepinephrine on OXT and OXTR mRNA expression were examined. After NE treatment, SCOV3 cells, OXTR expression was reduced by ~30%, and was not significantly changed in OVCAR8 cells. However, OXTR expression was significantly increased ($p<.01$) by fivefold after norepinephrine treatment in HEYA8 cells. OXT mRNA expression was not affected by NE treatment in SCOV3 and OVCAR8. Further, OXT mRNA was undetectable in HEYA8 cells. In the OV432 cell line mRNA for IL-6, OXTR and OXT was below the limit of detection.

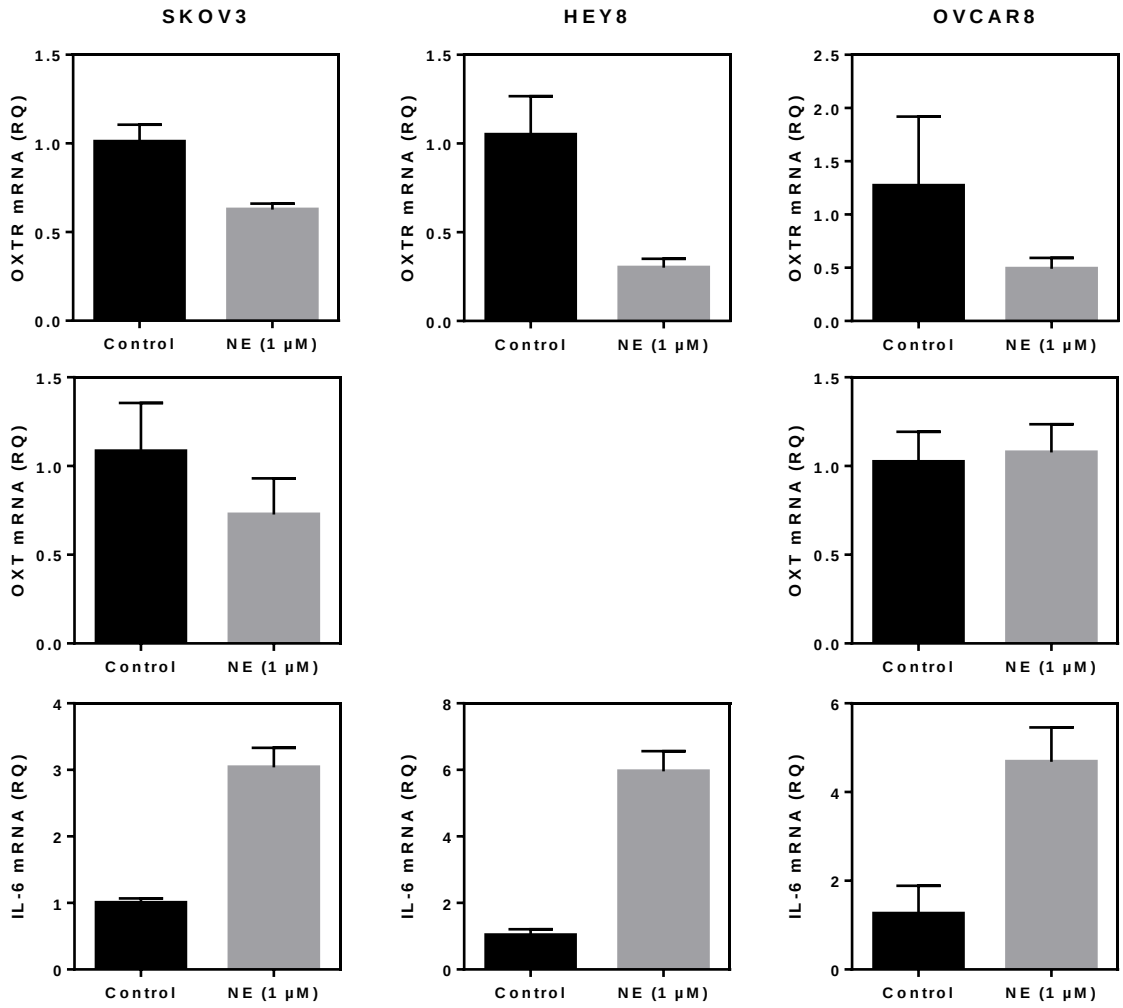

Figure 2: The ovarian cancer cell lines SCOV3, HEYA8, and OVCAR8 were grown to confluence and then incubated for 6 hours in the presence or absence of 1 um NE. After 6 hours the cellular mRNA was isolated and collected. mRNA was evaluated using Rt-PCR. Genes of of interest were normalized to 18s and compared to the control condition. Data is represented as fold change +/- SEM.

Effect of oxytocin on IL-6 secretion in catecholamine stimulated ovarian cancer cells.

Time course experiments in HEYA8 and SCOV3 cells showed that IL-6 secretion increased over time under control conditions and was further stimulated by norepinephrine. For later studies, the 6 hour time point was used since it represented the time point when stimulated IL-6 secretion was increasing linearly with norepinephrine treatment. Previous studies have shown that oxytocin inhibits proliferation of ovarian cancer [47], and OXT has been demonstrated to be anti-inflammatory *in vitro* and *in vivo* in various models of inflammation [65, 75, 77, 99]. To evaluate potential anti-inflammatory effects of oxytocin in the ovarian cancer cell lines, cells were treated with OXT in the presence or absence of norepinephrine for six hours and IL-6 secretion was measured. The glucocorticoid dexamethasone was used as a positive control for inhibiting inflammation [100]. Under control conditions, OXT treatment of cells reduced IL-6 secretion in SCOV3 and HEYA8, but the difference was not statistically significant. OVCAR8 cells treated with OXT alone showed no change of IL-6 secretion compared to the control (all $p>.05$). Incubation with norepinephrine (10 uM) significantly increased IL-6 secretion for all cell lines tested. The addition of OXT resulted in an attenuation of IL-6 secretion in norepinephrine-stimulated cells. Specifically, 1 nM oxytocin, in the presence of 10 µM norepinephrine, decreased IL-6 secretion by 54% in SCOV3 cells ($p < 0.05$). IL-6 secretion also decreased by 27%in HEYA8 cells ($p=.13$), and by 15% in OVCAR8 cells, although these did not reach statistical significance. Of note, OXT decreased IL-6 secretion at a substantially lower concentration than the positive control dexamethasone (1nM vs. 100 µM). OV432 did not secrete measureable amounts of IL-6 protein, so no further studies were performed with this cell line in this context.

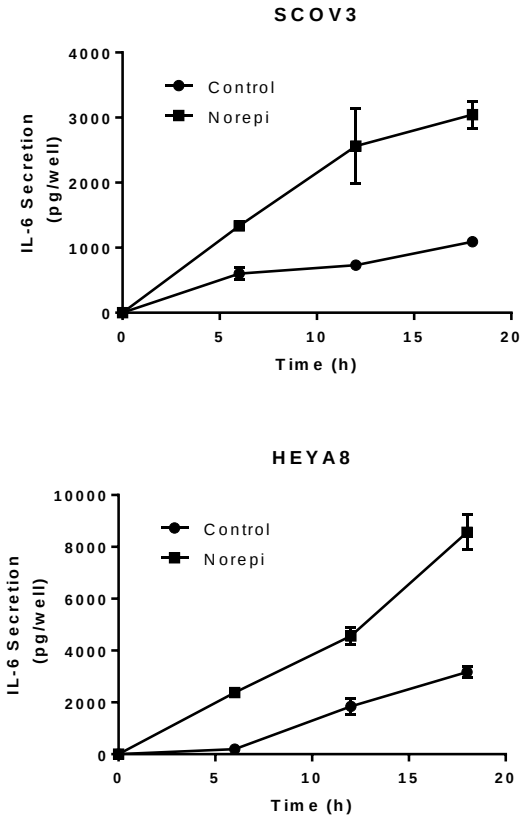

Figure 3: The ovarian cell lines SCOV3 and HEYA8 were grown to confluence and then incubated for 6, 12, or 18 hours alone or with the addition of 10 μm NE. After 6, 12, and 18 hours, respectably, the media was collected for measurement of IL-6 secretion. Data are representative of two-three experiments per cell line

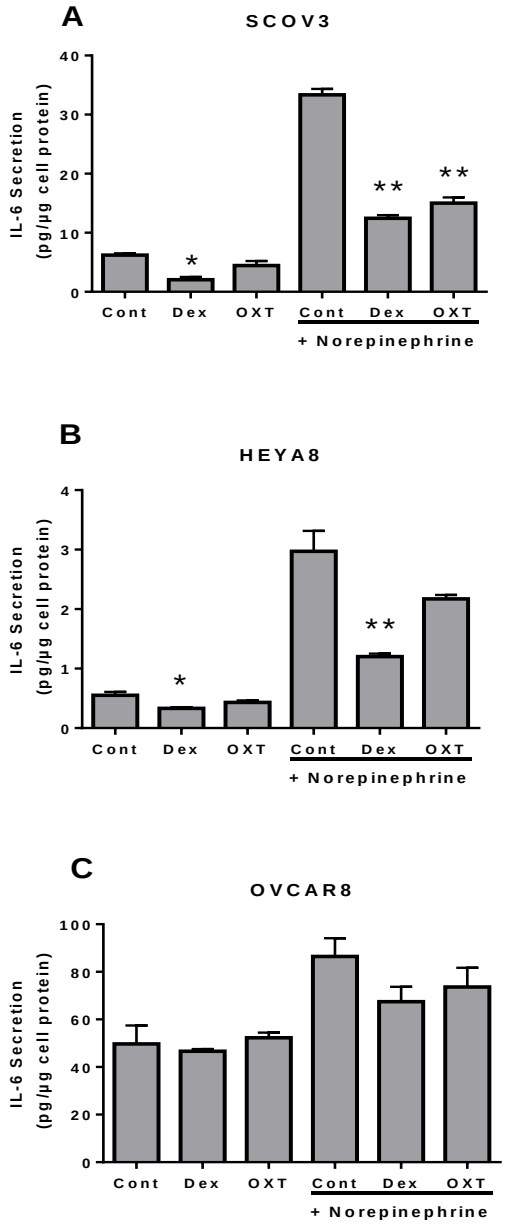

Figure 4: The ovarian cancer cells SCOV3, HEYA8, and OVCAR8 were grown to confluence and then incubated for 6 hours alone or with the addition of 10 µm NE with the conditions of 100 µm of dexamethasone or 1 nm of oxytocin. After 6 hours the media was collected for measurement of IL-6 secretion. Data are expressed as pg of IL-6 per µg of cell protein. Represented as +/- SEM, n=3 per condition. * denotes p<.05 compared to control condition. ** denotes p<.05 compared to control +NE condition. Data are representative of two-three experiments per cell line.

Effect of Prostaglandins on IL-6 and OXT secretion

Previous results have demonstrated that the prostaglandins PGE and PGF2α can have pro- or anti-inflammatory properties, respectively, in primary ovarian cell cultures [101] and that PGF2α can stimulate OXT release from these cells[102]. The present study evaluated the effects of these prostaglandins on IL-6 secretion, OXT secretion, and changes in mRNA of IL-6, OXT and OXTR in ovarian cancer cells.

Time course experiments in HEYA8 and SCOV3 cells revealed that PGE treatment of cells increased IL-6 secretion significantly in both cell lines ($p<.05$). When mRNA was evaluated after PGE-treatment of cells for 6 hours, it was observed that IL-6 mRNA expression increased twofold in each cell line ($p<.05$). In the HEYA8 cells, OXTR decreased by approximately 25% ($p>.05$), while in SCOV3 there was no change in OXTR by PG treatment. In addition, OXT secretion from these cells was also measured after PGE treatment. PGE did not effect OXT secretion in either cell line compared to control levels of secretion.

Results of the time course experiments in HEYA8 and SCOV3 displayed that treatment with PGF2α had no effect on IL-6 secretion. When mRNA was evaluated for the PGF2α treated cells, SCOV3 had a significant increase in OXT mRNA ($p<.05$), although this did not translate to increased OXT secretion. Further, IL-6 mRNA expression decreased by approximately 20%, though this value did not reach statistical significance. In HEYA8 cells, the PGF2α treatment had no effects on IL-6, OXT, and OXTR mRNA expression, as well as OXT secretion, compared to controls.

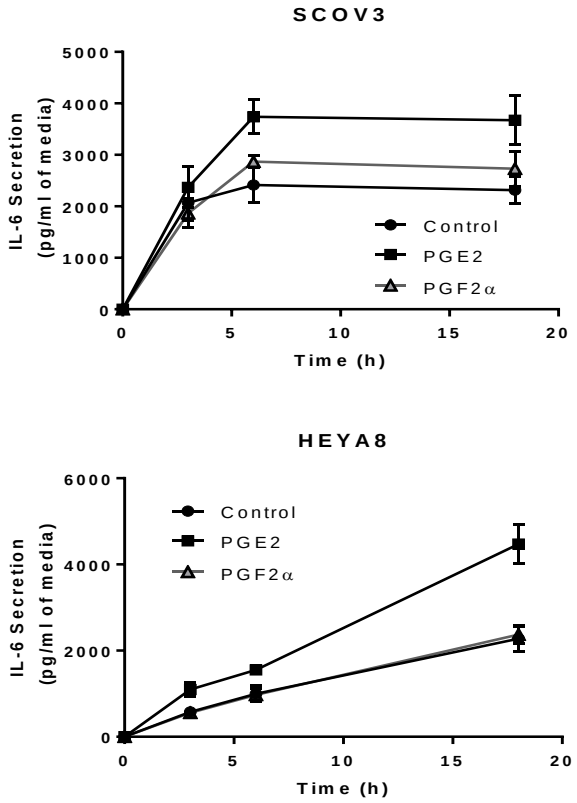

Figure 5: The ovarian cell lines SCOV3 and HEYA8 were grown to confluence and then incubated for 6, 12, or 18 hours alone or with the addition of PGE or PGF2α. After 6, 12, and 18 hours, respectably, the media was collected for measurement of IL-6 secretion. Data are expressed as pg of IL-6 per µg of cell protein. Represented as ≠ SEM, n=3 per condition. * denotes $p<.05$ compared to control condition. Data representive of two-three experiments per cell line.

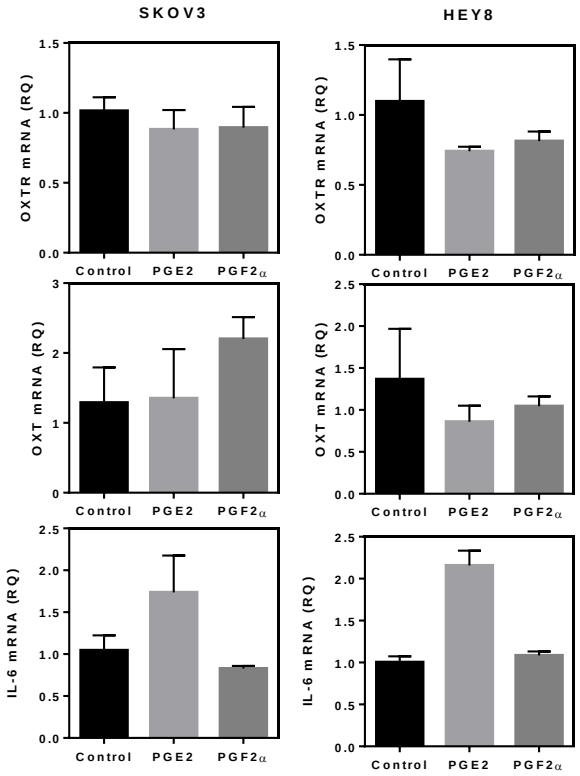

Figure 6: The ovarian cell lines SCOV3 and HEYA8 were grown to confluence and then incubated for 6 hours alone or with the addition of PGE, or PGF2α. After 6 hours the cellular mRNA was isolated and collected. mRNA was evaluated using rt-PCR. Genes of interest were normalized to 18s and compared to the control condition. Data is represented as fold change +/- SEM

CHAPTER FOUR: DISCUSSION

The current study was initiated by the observation that ascites fluid from subjects with ovarian cancer had elevated levels of oxytocin compared to plasma levels. Patients in the highest quartile of ascites fluid oxytocin were correlated (via a trend) to a lower cancer stage and improved survival at 5 years (Lutgendorf et al, personal communication). Based on those results we evaluated the OXT/OXTR pathway in several ovarian cancer cell lines to evaluate potential anti-inflammatory effects of OXT that could modulate cancer cell microenvironment.

This *in-vitro* study revealed that oxytocin has anti-inflammatory effects on high-grade serous ovarian cancer cell lines and that oxytocin decreased norepinephrine induced IL-6 secretion in these cells. By reducing the stress-induced inflammatory process in ovarian cancer cells, these data suggest that oxytocin may have a significant role in the ovarian cancer microenvironment.

Oxytocin receptor protein and mRNA were identified in ovarian cancer cell lines SCOV3, HEYA8, OVCAR8 and OV432, confirming previous results [47]. A novel finding was the demonstration of the discordance between mRNA and protein expression of OXT/OXTR in these ovarian carcinoma cells. This may be due to disparate regulation of the receptor, either at the mRNA or protein level. The association between mRNA and protein levels is still being investigated and indicates a complex regulation of the receptor. This phenomena provides an avenue for future research.

All four cell lines studied expressed an immunoreactive band of OXTR consistent with the known MW of the mature and immature (unglycosylated) forms of the receptor. Studies in uterine issues and results from our lab have previously identified different MW forms of the

oxytocin receptor [77, 103]. It is possible that different glycosylation sites on the receptor can have a role in ligand binding and affinity. While possible, other *in vitro* studies observed that the disruption of these glycosylation sites did not affect receptor localization to the plasma membrane or ligand binding to the receptor.

Inflammation resulting from stress plays an important role in ovarian cancer progression and angiogenesis[3, 18]. The current study examined the effects of oxytocin on specific aspects of this disease process. Oxytocin decreased norepinephrine stimulated IL-6 secretion by 54% in SCOV3 cells, 27%in HEYA8 cells, and by 15% in OVCAR8 cells. This is a novel finding, and is interesting in light of the fact that oxytocin receptor gene contains an IL-6 response element that upregulates the receptor in response to inflammation. This phenomenon has been observed in other cell types, including immune cells, suggesting that oxytocin may have a broader and an active role in the tumor microenvironment other than the ovarian cancer cells.

It has been reported that PGs can modulate inflammation and OXT secretion in normal/primary ovarian cells in culture, thus we evaluated whether PGS influenced OXT/OXTR and IL-6 in the ovarian cancer cell lines. Data showed that PGE2 induced IL-6 mRNA production and protein secretion. Neither PGE2, nor PGF2α altered OXT secretion or mRNA. These results suggest that OXT secretion in these cell lines respond differently to PGs than primary ovarian cells.

Studies in the past from our laboratory revealed that a stable social environment, characterized by increased affiliative social behavior, slowed the progression of atherosclerosis in an animal model [99, 104]. A more recent study showed that oxytocin receptors were present in endothelial cells, smooth muscle cells, monocytes, and macrophages, and that treatment with

oxytocin was responsible for significant decreases in oxidative stress and inflammation[77]. This suggests that oxytocin may slow the progression of atherosclerosis, the underlying inflammatory process in cardiovascular disease. Clinical studies for those diagnosed with cancer found that social support increased survival time/advantage. A similar anti-inflammatory mechanism appears to be present in ovarian cancer cells. There have been a substantial amount of studies that link social behavior to oxytocin, and the findings of the current study proposes that elevations in peripheral or local oxytocin in the ovarian cancer microenvironment, as a result of social environment, could work directly on ovarian cancer cells to slow the progression of the disease. Further studies will examine the relationship between mRNA and protein expression of OXT/OXTR, a pre-clinical model to examine oxytocin for potential pharmacological use, and further mechanistic studies to understand this unique phenomenon.

Future research could look into the male reproductive system. It has been speculated that oxytocin could be used as a biomarker for prostate cancer[105]. Similar to ovarian cancer patients, prostate cancer patients have serum and prostatic oxytocin levels that are higher than normal[105]. Further, because of the known effects of oxytocin in healthy pregnant women, future research could look at how this increase in oxytocin in ovarian cancer patients effects the individual in terms of pain, lactation, and other downstream effects caused by oxytocin. Currently, there is no pharmaceutical use for oxytocin aside from childbirth. Due to its anti-inflammatory effects in a number of models, oxytocin may be used more extensively in the future.

In summary, oxytocin receptors were identified in cultured ovarian cancer cells lines SCOV3, HEYA8, OVCAR8, and OV432. Norepinephrine induced IL-6 secretion was attenuated

in the cell lines by treatment with oxytocin. These novel findings suggest that oxytocin has the potential to be a pharmacological option in the future for ovarian cancer patients, to be a possible biomarker for ovarian cancer patients, and demonstrates the importance of social support being in the protocol for management of this disease.

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