LEPTIN’S ROLE IN PAIN IN FEMALES: EXPLORING LEPTIN IN A MOUSE MODEL OF ENDOMETRIOSIS

By

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A Thesis Submitted to The W.A. Franke Honors College
In Partial Fulfillment of the Bachelors degree
With Honors in

Physiology and Medical Sciences

THE UNIVERSITY OF ARIZONA

MAY 2022

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Acknowledgments

First, I would like to thank my senior Honors Thesis Advisor, Dr. Edita Navratilova of the Department of Pharmacology. Without her patience and efforts to guide me along the way in this research project, I would not have been able to complete my thesis. I would also like to thank the Porreca Lab members at the University of Arizona Department of Pharmacology for assisting me through the experiments necessary to complete this Honors Thesis. I would also like to thank Dr. Cindy Rankin for guiding me throughout my undergraduate Physiology career. Every Physiology student knows that if you know Dr. Rankin, you know everyone you need to know- a true testament to her initiative to connect all students with someone in her network who can help them. Finally, I would like to thank all the professors who have helped me along the way. I am profoundly grateful for the education I have received throughout my time at the University of Arizona, and I would not be the person and student I am today without each professor that has unwaveringly encouraged my success.
Abstract

Leptin, a hormone secreted by adipose tissue, is known for its relationship to energy homeostasis, metabolism, and obesity. However, leptin also has a pro-inflammatory role, and recently emerging research points to leptin as a contributor to pain. Chronic pain conditions have been the topic of studies for many years now, and it is well known that females are disproportionately impacted by these ailments. However, it is not fully understood why females are more likely to be affected by these chronic pain conditions. Due to estrogen, leptin levels are higher in women. Given that leptin is also potentially related to pain, introductory research studies have begun to explore the importance of leptin signaling in female pain specifically. Recent research has begun to explore the role of leptin in the disease endometriosis, which occurs in women and results in frequent, severe pain. Because leptin signaling participates in pain perception, a model of endometriosis serves as a useful study to understand the mechanism of female pain. This study explores the influence of leptin in the pain associated with endometriosis. This study is the foundation of experimental studies to determine the difference of leptin levels in endometriosis mouse models, paving the way for future studies to determine the potential relationship between increased leptin levels and pain in endometriosis and other chronic pain states impacting females.

Key Words: leptin, pain, endometriosis, women, females, chronic pain
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I. Introduction

Leptin and leptin receptors

Leptin, a 16 kDa, 167 amino acid product of the obese (ob) gene (Zhang et al., 1994), is one of the most known hormones secreted primarily by white adipose tissue (Kelesidis et al., 2010; Watanobe & Suda, 1999; Zhang et al., 1994). Leptin is secreted in a pulsatile fashion, with higher levels seen in the evening and early morning hours (Kelesidis et al., 2010). Leptin has several known functions in the body, but its most significant roles include energy homeostasis, neuroendocrine function, and regulation of metabolism and body weight by influencing food intake and energy expenditure (Kelesidis et al., 2010, Stephens et al., 1995; Halaas et al., 1995). As a hormone secreted by adipose tissue, leptin levels are strongly associated with adiposity. Plasma leptin concentration is directly related to amount of body fat in humans and rats (Kennedy et al., 1997; Rocha et al., 2001). Additionally, one study found that in females, leptin was higher in individuals with both chronic pain and obesity than those with chronic pain or obesity alone, suggesting that leptin signaling contributes to chronic pain and that this is exaggerated by increased adiposity (Hainsworth et al., 2021).

Leptin mediates its response by binding to specific leptin receptors or LEPRs (Figure 1), also known as ObRs, expressed in the brain and other peripheral tissues (Kelesidis et al., 2010). The leptin receptor is a member of the type I cytokine receptor family (Zabeau et al., 2003). Upon ligand binding, the leptin receptor dimerizes to mediate its action (Couturier & Jockers, 2003). Different isoforms of ObRs are the result of alternative splicing during the process of transcription. Each of the isoforms of ObR share identical extracellular and transmembrane domains, with intracellular domains of variable length characterizing the isoforms (Couturier & Jockers, 2003). The primary short isoform is known as ObRa, and this isoform transposes leptin
across the blood-brain barrier (Kelesidis et al., 2010). ObRb is the only long isoform leptin receptor that has clearly demonstrated signaling capacity and mediates signal transduction (Kelesidis et al., 2010; Zabeau et al., 2003). The long isoform is highly expressed in the hypothalamus, which is an important site for the regulation of energy homeostasis, demonstrating one way leptin influences energy and metabolism. The hypothalamus is also an important site for neuroendocrine function, which may provide a clue as to how leptin influences pain (Kelesidis et al., 2010).

![Figure 1: Leptin is secreted by adipocytes and binds to the leptin receptor (LEPR or Ob-R). Leptin may bind to different isoforms of the leptin receptor in different areas of the body, including long and short isoforms (ObRa, ObRb, etc.).](image)

The binding of leptin (LEP) to leptin receptor (LEPR) activates signal transduction pathways, including the JAK-STAT3 pathway, which is important for the regulation of energy homeostasis, the PI3K pathway important for regulation of food intake and glucose homeostasis, and more pathways including MAPK, AMPK, and mTOR.

While each of these pathways are significant in leptin’s signaling for various functions, the JAK-STAT pathway is the pathway hypothesized to be the mechanism for leptin’s role in pain as depicted in Figure 2.
Figure 2: Action of leptin and LEPR in the activation of JAK/STAT pathway to perform various functions in the body such as energy balance and including its hypothesized role in pain.

Leptin functions

Most known for its connection to obesity as an appetite regulatory hormone, one of leptin’s greatest roles is energy homeostasis. Leptin functions as what is known as an “adipokine;” a cell-signaling molecule secreted by adipose tissue that has energetic and metabolic influences in the body (Kelesidis et al., 2010). LEP acts via ObRb binding in the hypothalamus, where it activates a complex neuronal circuit involving appetite diminishing and appetite-stimulating neuropeptides to control food intake (Kelesidis et al., 2010). In the fed state, leptin is secreted by adipose tissue and binds Ob-R activating anorexigenic or appetite-suppressing proopiomelanocortin producing neurons and inhibiting orexigenic or appetite-stimulating neuropeptide Y/agouti related peptide (NPY/AgRP) producing neurons in the arcuate nucleus (Ramirez & Claret, 2015). The result is a net appetite suppressing effect via the hypothalamus, leading to decreased food intake. Furthermore, leptin also interacts with the
mesolimbic dopamine system responsible for motivation and reward of feeding to contribute to feelings of satiety. Leptin’s role in energy balance and eating behaviors demonstrates an important connection with pain. Obese individuals are a group that is known to have higher levels of leptin. Furthermore, obese individuals are at increased risk for chronic pain conditions. Thus, the increased levels of leptin may lead to heightened pain, and researchers are exploring the mechanism of leptin-induced pain in various conditions.

One proposed way leptin influences pain is through inflammation induced by the hormone. Leptin is a pro-inflammatory adipokine, and emerging research demonstrates a connection between leptin-mediated inflammation and increased pain sensitivity (Younger et al., 2016). Leptin’s role in several pain conditions has been explored in recent research. These studies have shown that leptin may sensitize nociceptors (Alvarez et al., 2014), leptin is associated with body pain and may be a driver of generalized pain states (Younger et al., 2016), elevated leptin concentrations may affect pain levels in conditions such as osteoarthritis (Askari et al., 2020; Lubbeke et al., 2013), and that leptin can be used as a marker for endometriosis severity, demonstrating a tie between leptin and pain in women (Alvarez et al., 2014; Hussein et al., 2020). Research is beginning to explore leptin’s role in pain, with promising connections being demonstrated.

One study explored the potential effect of blocking leptin signaling through the JAK2/STAT3 pathway in a rat model of endometrial pain. In this study, researchers blocked leptin action through a JAK2 inhibitor, thereby inhibiting LEP/Ob-R signaling (Alvarez et al., 2014). Injection of the JAK2 inhibitor led to significantly reduced mechanical hyperalgesia, or an exaggerated response to a painful mechanical stimulus (Alvarez et al., 2014). Alvarez et al. assessed mechanical hyperalgesia by quantitatively measuring mechanical nociceptive threshold
in the ectopic endometrial lesion of rats using a manually driven force transducer (Alvarez et al., 2014). The lowered mechanical hyperalgesia following JAK2 inhibition suggests that LEP/Ob-R action via the JAK2/STAT3 pathway contributes to pain (Alvarez et al., 2014). Figure 3 demonstrates the proposed function of leptin in pain via the JAK/STAT pathway.

**Figure 3: Leptin’s hypothesized role in pain (mechanical hyperalgesia) via the JAK/STAT pathway in Alvarez study (left). JAK2 inhibition led to significantly attenuated mechanical hyperalgesia suggesting a hypothesized role for leptin action via the JAK/STAT pathway in pain.**

**Role of leptin in preclinical models of pain**

While research has demonstrated that there is a connection between leptin and pain, the mechanism of leptin-induced pain is not fully agreed upon. Researchers hypothesize that leptin increases pain sensitivity through its proinflammatory action, proposing several mechanisms for leptin-induced inflammation. Namely, leptin upregulates IL-18 secretion in human monocytes through caspase-1 activation to promote inflammation (Jitprasertwong et al., 2014). Another study suggests that leptin induces neuropathic pain through the stimulation of macrophages and the release of pronociceptive factors in mice (Maeda et al., 2009). Studies also demonstrate that leptin increased IL-1β using microglia as mediators in rats (Lafrance et al., 2010), which is being
considered as a trigger of chronic pain states (Younger et al., 2016). While the exact mechanism for leptin-induced pain is not agreed upon in literature, many studies have demonstrated promising findings for a role of leptin in pain.

According to one study, the leptin receptor Ob-R is expressed in afferent pathways contributing to pain processing such as the dorsal root ganglion (DRG) neurons (Chen et al., 2006, Murphy et al., 2013), as well as in neurons and glia in the superficial dorsal horn of the spinal cord (Alvarez et al., 2014). Studies have also shown that leptin induces mechanical allodynia and contributes to neuropathic pain and that leptin receptor antagonists inhibit nerve injury induced mechanical allodynia in a rat model of the condition chronic constriction sciatic nerve injury or CCI (Lim et al., 2009). Furthermore, Ob-R or leptin null animals (ob/ob mice) fail to develop neuropathic pain-like behaviors in mice with partial sciatic nerve ligation or PSL, suggesting that leptin signaling is important in the pain pathway related to chronic pain (Maeda et al., 2009).

*Leptin in chronic pain conditions in humans*

After this established connection between leptin and pain, studies have begun to explore leptin’s role in specific chronic pain conditions. Migraines are frequently studied in today’s research, notably mentioning the prevalence of this chronic pain condition in females. As discussed above, leptin influences POMC and AgRP/NPY neurons in the arcuate nucleus in its feeding regulation effects. The arcuate nucleus also projects to other hypothalamic nuclei including the paraventricular nucleus (PVN), dorsomedial nucleus (DMH), lateral hypothalamic nuclei (LH), and ventromedial hypothalamic nuclei (VMH); all being areas that are known to function in migraine pathophysiology. Studies have shown disturbances in leptin levels in chronic sufferers of migraines (Goadsby et al., 2017). Furthermore, migraines are more common
in women, and drastically so: women are 2-3 times more likely to experience migraines. Obesity is known to worsen migraines as well as other pain disorders, and because obesity is associated with higher levels of fat, obesity also results in elevated leptin levels (Rocha et al., 2001). This correlation poses an important question as to if leptin may be contributing to this increased pain.

Additionally, researchers explored leptin’s role in osteoarthritis, another condition resulting in frequent pain for those suffering from the disorder. Leptin was established as a key link between obesity and osteoarthritis (OA) and may help to explain the joint pain that is a prominent clinical feature of OA (Lubbeke et al., 2013). One study demonstrated that weight reduction decreases leptin levels and simultaneously resulted in decreased reported joint pain in patients with OA (Christensen et al., 2005; Miller et al., 2008). Furthermore, higher OA pain levels are reported in women and obese patients, both groups which are known to have higher leptin levels (Greenspan et al., 2007), further establishing a possible connection between leptin and pain in OA. Another study demonstrated that high synovial fluid concentrations of leptin resulted in a substantial increase in reported OA pain and the increased levels of pain in women and obese patients were associated with high leptin concentrations; however, causality could not be established between leptin and pain due to the design limitations of the study (Lubbeke et al., 2013). Finally, it was found that higher leptin was associated with higher BMI and a higher OA score, alleviated by exercise to decrease adiposity and leptin levels. However, this study also demonstrated several limitations and after controlling for other variables found that leptin levels did not have a significant relationship with pain levels (Askari et al., 2020). Therefore, more research is needed to establish what leptin’s function is in OA pathogenesis and pain.
Key findings in research demonstrate leptin’s role in pain, pointing to differences in leptin levels between males and females, as well as differences in pain experienced between males and females. It is this area of research that the current study aims to focus on.

The role of leptin in female pain

The higher incidence of chronic pain conditions in females is well known in research, with research pointing to sex hormones as the primary reason for this difference. However, the actual mechanism for increased pain in females is still unclear (Vacca et al., 2021). Studies have shown that the immune system – and its sex differences – is crucial in the expression, progression, and maintenance of chronic pain (Vacca et al., 2021). Given that leptin circulates as a pro-inflammatory adipokine, it is important to consider if it may have a potential role.

Estrogen, a female-prominent hormone, promotes metabolism, accumulation, and distribution of adipose tissue (Mayes & Watson, 2004). Like the hormone leptin, estrogen causes reduction in food intake, and ovariectomized female mice demonstrated moderate reduction in body weight (Pelleymounter). Given estrogen’s connection to adipose tissue, estrogen is also importantly connected to the hormone leptin. Estrogen stimulates leptin mRNA expression and leptin secretion from adipose tissue (Fungfuang et al., 2013). Estrogen also upregulates the expression of Ob-R in DRG neurons in the pain pathway (Chen et al., 2006). Because estrogen is a primary female hormone and estrogen stimulates leptin secretion, women have naturally higher occurring levels of leptin in non-diseased states as compared to men (Demerath et al., 1999; Saad et al., 1997). Sex steroid hormones have major effects on leptin production in humans and rats (Pinilla et al., 1999; Watanobe & Suda, 1999). Given leptin’s
connection to increased pain sensitivity, it is possible that leptin may help explain the increased pain that women experience, though demonstration of the mechanism requires more research.

Several studies have further strengthened the idea that leptin may be related to female prevalence in most pain conditions. One study found that daily fluctuations in serum leptin were predictive of fatigue severity in women with chronic fatigue syndrome; on days of higher leptin concentrations, women reported higher fatigue (Younger et al., 2016). In this study, leptin was the most consistent in association with fatigue severity as compared to fifty other cytokines and chemokines (Younger et al., 2016). Chronic fatigue syndrome often overlaps with fibromyalgia, a chronic disease characterized by widespread musculoskeletal pain and sensitivity to mechanical pressure in soft tissue that disproportionately impacts women (Younger et al., 2016). This led these researchers to explore if leptin may be related to the widespread musculoskeletal pain seen in women with fibromyalgia. Younger et al. completed two studies exploring leptin’s relationship to pain in fibromyalgia: first, a small pilot longitudinal study, followed by a large cross-sectional study. In the pilot study, three women with fibromyalgia had blood draws for twenty-five consecutive days and self-reported their musculoskeletal pain on each of these days. The results showed that daily fluctuations of leptin were positively associated with pain in all three participants (Younger et al., 2016). In the cross-sectional study, researchers took single blood draws to determine leptin levels and participants self-reported body pain. The results once again demonstrated that leptin was associated with self-reported pain, with higher leptin levels being associated with greater pain both within and between individuals (Younger et al., 2016). Overall, the researchers found that one of the effects of increased systemic leptin may be greater sensitivity to pain. Although, the study was not designed to determine causality (Younger et al., 2016), it suggests that leptin may be a driver in pain in fibromyalgia. These results suggest that it
is worthwhile to explore leptin’s role in other chronic pain diseases, specifically those that primarily impact women, such as the disease endometriosis.

The role of leptin in endometriosis

While the above details how leptin’s role in pain has been explored in research, only few studies have explored leptin’s role in pain in endometriosis specifically. Initially defined as the surgical detection of endometrial tissue outside of the uterine cavity, depicted in Figure 4 (Sampson, 1927), endometriosis is often difficult to diagnose except by surgical intervention (Giudice & Kao, 2004).

![Endometriosis diagram](image)

**Figure 4: Endometriosis is a diseased characterized by the presence of endometrial tissue located in abnormal locations outside of the uterus, including but not limited to on the ovaries, fallopian tube, within the cavity, etc.**

These ectopic endometrial tissues respond to hormones similarly to how the intrauterine endometrium does, resulting in a response during the menstrual cycle. Because the endometrial tissue acts like the endometrium, it grows and responds to the hormone estrogen (Bulun et al., 2019). This dependence results in the prevalence of the disease during reproductive years,
especially between ages 30 to 45. A prime symptom of endometriosis is pain during menstruation, sex, and urination, often connected with infertility (Bulun et al., 2019). The different forms of debilitating pain that characterize this disease are a major clinical issue, as women with these symptoms report troubled mental health and an overall lower quality of life (Fattori et al., 2020).

The true character of the disease endometriosis encompasses much more than endometrial tissue outside of the uterus, including issues with fertility, frequent recurrence of symptoms (Bulun et al., 2019), and key clinical features such as intense primary dysmenorrhea or painful periods (Brosens et al., 2013), debilitating menstrual pelvic pain experienced continually or intermittently until menopause, etc. as demonstrated in Table 1 (Brawn et al., 2014). Endometriosis is characterized by an estrogen-dependent chronic inflammatory process that mainly affects pelvic tissues (Bulun et al., 2019). The inflammation driving endometriosis is primarily driven by and dependent on the hormone estrogen (Han et al., 2015; Noble et al., 1996; Noble et al., 1997). Pelvic endometriosis- which describes most endometriosis cases occurs primarily through retrograde menstruation or the repeated retrograde travel and survival of shed endometrial tissue in the lower abdominal cavity (Bulun et al., 2019).

### Table 1: Key clinical features of endometriosis.

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<th>Key Clinical Features of Endometriosis</th>
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<tr>
<td>Presence of endometrial tissue outside of the uterus</td>
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<td>Menstrual pelvic pain</td>
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<tr>
<td>Intense primary dysmenorrhea</td>
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<tr>
<td>Painful urination during menses</td>
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<tr>
<td>Infertility</td>
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Over the years, researchers proposed various mechanisms for the development of endometriosis (Table 2). The first and most supported hypothesis was from Sampson, suggesting that fragments of menstrual endometrium pass through fallopian tubes and implant on peritoneal surfaces (Sampson, 1927). Sampson also hypothesized that menstrual tissue from the endometrial cavity reaches the pelvic cavity or other distant body sites via veins or lymphatic vessels, but this hypothesis has not received adequate support in contrast to the first hypothesis (Sampson, 1927). Furthermore, Ferguson and others hypothesized that the peritoneum undergoes metaplasia and differentiates into endometriotic lesions within the peritoneal cavity, resulting in endometriosis (Ferguson et al., 1969). Finally, another hypothesis suggests that circulating blood cells originating from the bone marrow differentiate into endometriotic tissue at various body sites (Sasson & Taylor, 2008). While there are several proposed mechanisms, most all recent relevant data support Sampson’s main postulate as the mechanism for endometriosis (Bulun et al., 2009).

Table 2: Proposed mechanisms of the development of endometriosis in current literature.

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<th>Proposed Mechanism</th>
<th>Support</th>
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<tr>
<td>Sampson et al. (1927)</td>
<td>Fragment of menstrual endometrium pass through fallopian tubes and implant and persist on peritoneal surfaces</td>
<td>Supported by most molecular and clinical data accumulated in recent research as the main mechanism for pelvic endometriosis Demonstrated in primate models Observed naturally in humans Spontaneous endometriosis occurs exclusively in species that menstruate</td>
</tr>
<tr>
<td>Ferguson et al. (1969)</td>
<td>Peritoneum undergoes metaplasia to differentiate into islands of endometriotic lesions within the peritoneal cavity</td>
<td>Challenging to construct clinically relevant models to test theory; not generally supported</td>
</tr>
<tr>
<td><strong>Sampson et al. (1927)</strong></td>
<td>Menstrual tissue from endometrial cavity reaches pelvic cavity or other distant body sites via veins or lymphatic vessels</td>
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<tr>
<td><strong>Sasson et al. (2008)</strong></td>
<td>Circulating blood cells originating from bone marrow differentiate into endometriotic tissue at various body sites</td>
<td>Challenging to construct clinically relevant models to test theory; not generally supported</td>
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As mentioned, estrogen is a key hormone in endometriosis progression as it enhances the survival or persistence of ectopic endometriotic tissue (Ryan & Taylor, 1997), (Bruner et al., 1997). Furthermore, prostaglandins and cytokines mediate pain and inflammation (Ryan & Taylor, 1997), (Bruner et al., 1997). The inflammation and estrogen production in endometriosis are linked by a positive feedback cycle in which chronic overexpression of aromatase and COX2 support sustained production of estradiol and PGE2 in endometriotic tissue (Tsai et al., 2001). Aromatase inhibitors that disrupt estradiol biosynthesis reduce endometriosis-associated pain and cause regression of pelvic lesions demonstrating the key role of estrogen in endometriosis progression and pain (Attar & Bulun, 2006; Takayama et al., 1998).

The lesions characteristic of endometriosis are hypothesized to be induced by retrograde menstruation, during which damage-associated molecular patterns (DAMPs) can be released and activate immune cells such as macrophages (Fattori et al., 2020). These activated immune cells then produce proinflammatory mediators such as TNF-a, IL-1B, and IL-33, and contribute to pain. Nociceptor neurons also further increase and maintain inflammation by secreting neuropeptides such as substance P and calcitonin gene-related peptide (CGRP) (Fattori et al., 2020). While researchers believe that proinflammatory mediators lead to pain in endometriosis, other factors are also secreted that may lead to this pain such as adipose tissue secreted factors.
Leptin is one adipose tissue secreted factor reported to affect afferent nerves, and as a pro-inflammatory adipokine, leptin may contribute to the pain characteristic of endometriosis by acting on the afferent nerves in sensory processing (Figure 5).

Figure 5: Adipose tissue secreted factors reported to affect afferent nerves (https://europepmc.org/article/med/30733616).

Endometriosis affects 5% to 10% of reproductive-age women in the United States (Bulun et al., 2019). Given the connections of leptin with other chronic pain conditions, it is of interest to explore whether leptin may be influencing chronic pain within endometriosis as well.

Specifically, because leptin has immune-regulatory and proinflammatory functions, and because recent reports state its function in the female reproductive system, it was suggested that leptin signaling contributes to the pathogenesis of endometriosis (Hussein et al., 2020).

Hussein and colleagues explored leptin’s potential role in a cross-sectional study consisting of thirty women in the control group and thirty women with endometriosis, classifying the severity of endometrial lesions in stages I-IV according to the American Society for Reproductive Medicine. This study demonstrates that serum leptin was much higher in the endometriotic group than in the control group and that the serum leptin levels increased with
endometriosis progression. These results suggest that leptin is involved in endometriosis (Hussein et al., 2020), though it is not enough to determine causality of leptin in endometriosis etiology and pain. Not only has leptin been shown to be present in higher levels in endometriosis, but growing evidence also suggests a role for leptin in the establishment of lesions in endometriosis and the expression of symptoms related to endometriosis (Barcz et al., 2008; Bedaiwy et al., 2006; Styer et al., 2008; Wu et al., 2007). One study found that mice lacking functional Ob-R did not develop endometriosis-like lesions and that Ob-R antagonist inhibits lesion establishment and development (Styer et al., 2008), suggesting a key role for leptin in the development of endometriosis. However, these studies did not specifically look at leptin’s relationship to pain in endometriosis. Given leptin’s connection to pain in other disease states such as OA and neuropathic pain, it is possible that leptin not only contributes to the progression of endometriosis as hypothesized by the researchers but may also influence pain associated with endometriosis.

Further studies worked to explore how leptin may lead to chronic pain in a rat model of endometriosis. In this endometriosis rat model, Alvarez et al. anesthetized rats and excised one cm of the right uterine horn that they implanted on the gastrocnemius muscle of the same rat. Researchers hypothesized that ectopic endometrial lesions produce leptin which leads to mechanical hyperalgesia by acting on nociceptors innerving the lesion, and that this sensitivity is dependent on estrogen. With this hypothesis in mind, the study aims to explore how targeting leptin-signaling may be useful for the treatment of endometriosis (Alvarez et al., 2014). First, the study agreed with prior studies in that leptin expression in ectopic endometrium was higher than in eutopic endometrium (Alvarez et al., 2014). The study found that there was a significant decrease in the mechanical nociceptive threshold at the site of the implant in the endometrial
model (Alvarez et al., 2014), and further found that endometriotic-like cystic lesions exhibit increased leptin mRNA and protein. Treatment with intralesional injection of an Ob-R antagonist at the site of the implanted uterine tissue to inhibit the action of leptin inhibited lesion establishment (Alvarez et al., 2014), though researchers believe this is through local effect. The study found that intramuscular injection of leptin in naïve rats resulted in dose-dependent mechanical hyperalgesia, demonstrating that leptin is related to increased pain (Alvarez et al., 2014). Additionally, the study demonstrated that local leptin injection also produced persistent nociceptor sensitization to stimuli, suggesting that leptin can sensitize nociceptors (Alvarez et al., 2014). The study ultimately concluded that estrogen was necessary for full expression of acute and persistent leptin-induced mechanical hyperalgesia (Alvarez et al., 2014), but that leptin released from endometrial lesions acts locally to produce mechanical hyperalgesia. The findings of this study are important because they suggest that with further research, therapeutic interventions targeting leptin actions might be useful for the treatment of pain in endometriosis.

Pelvic pain associated with endometriosis is currently managed by suppression of ovulatory menses and estrogen production inhibitors or via surgical removal of pelvic lesions (Bulun et al., 2019). As the pathophysiology of endometriosis becomes increasingly understood, new treatments are becoming available. While suppression of hormones and ovulatory menses are the current treatments for endometriosis and alleviate pain associated with the syndrome, it is important to further understand the mechanism behind pain in endometriosis as some females prefer not to alter their estrogen levels in this way. Furthermore, a deeper understanding of what causes pain in endometriosis may lead to discovery in female pain within other disorders. Thus, exploring leptin signaling as a mechanism for pain in endometriosis is a worthwhile area of study.
An endometriosis mouse model serves as a strong model for researching female pain. Endometriosis has a multifactorial etiology and high prevalence, resembling other chronic inflammatory disorders associated with pain that may be of interest when studying differences in male pain versus female pain (Bulun et al., 2019). Animal models are useful to investigate mechanisms and causal relationships, giving the ability to control for variables that complicate studies in patients and allowing for the use of experimental drugs.

**Methods**

**Animals**

For the experiments, adult female C57BL/J mice were used. The animals were housed in the animal care facility at the University of Arizona under environmentally controlled conditions with food and water available. All animal care and experimental use conformed to NIH guidelines (NIH Guide for the Care and Use of Laboratory Animals). Care was taken to minimize all suffering of laboratory animals.

**Endometriosis Mouse Model**

To induce endometriosis, subcutaneous injection of 3 microg/mouse estradiol benzoate was performed to stimulate the growth of the endometrium in donor mice. Next, surgical removal of the uterus from the donor mouse was completed 7 days after estradiol injection. The uterus of the donor mouse was dissected and split. The uterine horns were collected and minced in Hanks balanced salt solution. 500 µl of minced uterine horn tissue was injected intraperitoneally into the recipient mice. Sham mice received an injection of solely 500 µl Hanks balanced salt solution. The current study analyzes tissues of mice from this endometriosis model.
Assessment of pain in endometriosis mouse model

To measure pain in endometriosis model mice and sham mice, tactile frequency of response to stimulus applied mouse abdomen was measured over time following lesion development in the endometriosis group. Pain was measured by probing the mouse's abdomen with a von Frey filament ten times, noting how many times out of ten the mice have a pain response. Animals with abdominal sensitivity, or abdominal allodynia, respond more often as compared to sham mice. The percent of response was plotted as a function of time after uterine tissue implant.

Experimental Design

As a foundational pilot study, leptin and leptin receptor levels were measured in endometrial lesions from four mice from the endometriosis mouse model group and compared to levels observed in uterine tissue from naïve control mice. LEP and LEPR levels were measured using Western Blot technique (leptin and leptin receptor) and/or ELISA (leptin). Furthermore, imaging of both naïve uterine tissue and endometriotic lesion tissues was done, both being visualized for leptin and leptin receptor. Finally, a statistical analysis (unpaired t-test, p < 0.05) was done to compare leptin and leptin receptor levels in naïve mouse endometrium and endometriosis mouse model tissues.

Tissue Collection

Adult female mice were sacrificed in accordance with ethical guidelines and control uterus tissues were collected via anatomical dissection. The experimental tissues were collected
from the recipient mice in the endometrial mouse model. Tissues were kept frozen until ready for use in experiments.

*Western Blotting*

Western blot testing was performed to determine if there is a difference in leptin and leptin receptor levels for endometriosis model mice as compared to naïve mice. Western blot was run using control uterine tissues and endometriotic lesion tissues from the mouse endometriosis model to determine the presence and levels of both LEP and LEPR. Tissue was lysed on ice in lysis buffer containing protease inhibitor, phosphatase inhibitor and nuclease. Protein concentration was determined in each sample using a BCA protein assay kit (Bio-Rad). 19 µl of sample protein was used for SDS-PAGE. Antibodies used were, respectively, leptin antibody SAB2101335 (1:1000), leptin receptor antibody CUS-PA009807 (1:1000), tubulin: MA5-16308 (1:5000). Immunoreactive bands were visualized by enhanced chemiluminescence and exposure to film. Blots were imaged using Odyssey imager (LI-COR) and analyzed by Image studio software.

*ELISA*

ELISA was run using control tissues to determine the presence and levels of leptin in dissected mouse uterine tissues using leptin mouse ELISA Kit Invitrogen KMC2281. Next, ELISA was performed using endometriosis model mouse lesion tissue to determine the presence and levels of leptin in the endometriosis disease state (leptin mouse ELISA Kit Invitrogen KMC2281).
Statistical analysis

Unpaired t-test was used to perform a statistical analysis comparing leptin and leptin receptor levels in uterine control tissues and endometriosis model lesions using data from Western blots for leptin and leptin receptor and from leptin ELISA (p<0.05).

Results

Results of the endometriosis mouse model (Figure 6) done in Porreca Mayo Clinic Laboratory demonstrate increased pain response in mice in the endometriosis group as compared to sham mice. Abdominal allodynia is demonstrated in mice in the endometriosis group with confirmed lesions, with an increased response to abdominal probing at about six out of ten times, as compared to about two out of ten times in controls.

Figure 6: Tactile frequency of responses over time in murine endometriosis model. Control mice (n=9) demonstrated a low tactile frequency of response over time, demonstrating baseline pain response. Endometriosis mice (n=26) demonstrated an increase tactile frequency of response over time, indicating an increased pain response over time that may be characteristic of progressive endometriosis.
Following completion of Western Blots, leptin was detected at an expected molecular weight around 15 kDa (Figure 7). These results demonstrate a control and confirm that leptin is detectable in uterine tissues in mice. Furthermore, imaging detected leptin in both naïve mice and endometriosis mice, with higher levels of leptin in endometriosis mice (Figure 9).

In preliminary experiments, we detected different isoforms of leptin receptor in both naïve mice and endometriosis mice, with higher levels of ObRe isoform in endometriosis mice* (Figure 8). Completion of statistical analysis comparing leptin levels in naïve mice and endometriosis mice implied that endometriosis mice have higher levels of leptin (Figure 10, Figure 11), but these results were not statistically significant (p<0.05) due to the small sample size. Furthermore, Figure 10 demonstrates that statistical analysis comparing leptin receptor levels from Western blots in naïve mice and endometriosis mice demonstrated that ObRe levels are higher in endometriosis mice* (n=4). Finally, naïve endometrium has higher level of ObRa compared to endometriosis lesion (Figure 8).

**Figure 7: Detection of leptin (around 15kDa) via chemiluminescent detection. Leptin is a 16 kDa hormone.**
Figure 8: Detection of various isoforms of leptin receptor in naïve endometrium and endometriosis model.

Figure 9: Naive uterus and endometriotic lesions stained for leptin. A) Presence of leptin in naive murine uterine samples. B) Presence of leptin in endometriotic lesions in murine model of endometriosis. C) Presence of leptin in endometriosis lesions in murine model (20x). Leptin
levels are visibly higher in the endometriosis model, which agrees with the findings of prior studies. D) Presence of LepR in mouse endometrium.

Figure 10: A) Western blot results for leptin in naive uterus and endometriotic lesions. No significant difference was found between the two. B) Western blot results for leptin receptor in naive uterus and endometriotic lesions. Leptin receptor (ObRe) levels were found to be higher in endometriotic lesions as compared to naive murine uterine samples*. 

N=4
Figure 11: ELISA results for leptin in naive murine uterine samples and endometriotic lesions from murine model of endometriosis. No significant difference was found in leptin levels between the two groups.

Discussion

The findings of this study serve as the basis for future studies investigating the mechanisms of leptin mediated sensitization and pain in endometriosis. The current preliminary study demonstrates that leptin receptor levels are higher in the endometriosis model*, and that leptin appears to be higher, though more animals are needed to be included to confirm these findings. Increasing the sample size will help determine the generalizability of these initial results. With this in mind, we can continue exploring the role that this increased leptin may have in endometriosis and the pain associated with this disease. Given the disease’s prevalence, such findings could eventually lead to helping millions of women worldwide struggling with pain in endometriosis.
Potential Therapeutic Strategies

Given the proposed role of leptin signaling in pain, research needs to explore the mechanism of how leptin increases pain sensitivity to target leptin as a therapeutic strategy for pain. Leptin levels are closely connected with adipose tissue and obesity, therefore, reducing leptin levels through calorie restriction, weight reduction, and/or physical activity is one strategy that could help alleviate pain (Younger et al., 2016). Another strategy may be leptin antagonists as one study demonstrated that leptin receptor antagonists inhibited hyperalgesia in endometriosis (Alvarez et al., 2014), though the side-effects of these antagonists pose difficulty in this strategy of pain reduction except in local use (Younger et al., 2016). Furthermore, targeting leptin as it modulates microglial activity may be beneficial in future pain therapies (Younger et al., 2016).

Specifically looking at the disease endometriosis, as of 2020, there were still a lack of accurate models that capture the disease phenotype for endometriosis, leading to difficulty in testing solutions for the pain associated with the disease (Fattori et al., 2020). The first step to alleviating the chronic pain that many women experience with endometriosis is developing a working model and using the model to identify a root to this cause of pain. The developed murine model of endometriosis is a tool that can be used to further explore the potential mechanisms of the pain in endometriosis that could be targeted to effectively treat this pain.

To continue the current study, the murine endometriosis model can be used to measure pain in naïve and endometriosis mice. Simultaneously measuring leptin levels would allow us to discover if leptin levels are higher with increased pain responses, indicating a potential role for leptin in pain in endometriosis. Furthermore, as levels of leptin differ with a female’s cycle, the
researchers must control for the time of the cycle mice in both the control and experimental groups.

**Conclusion**

This introductory study explores the research covering leptin’s role in pain, specifically in females and in endometriosis. The results of the experiments completed for this study agree with findings of prior studies, including Hussein and Alvarez, that leptin levels are higher in endometrial lesions as compared to naïve uterine tissue, validating our mouse model of endometriosis. Further, we started to explore the expression of leptin receptor isoforms in endometrial lesions, which play significant roles in activation of leptin signal transduction pathways promoting pain.

Given that women experience chronic pain at a significantly higher rate than their male counterparts, it is important to continue studying sex differences in pain to better understand and treat chronic pain conditions for both sexes. Though endometriosis is just one example of chronic pain afflicting women, research must explore other chronic pain disorders to lead to therapeutic strategies to deal with chronic pain.

With further research, particularly involving the mechanisms and signaling pathways of leptin-induced pain, therapeutic interventions targeting leptin actions might be useful for the treatment of endometriosis and other chronic pain conditions. However, it is important to note the difficulty that would come with targeting leptin. Leptin is an extremely important hormone with effects in control of food intake, metabolism, energy, and even the immune system. Targeting leptin would require significant, proper research to avoid interference with these other important systems in the body.
References


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