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The Influence of Oxytocin on Adipose Tissue, Inflammation and Atherosclerosis

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UNIVERSITY OF MIAMI

THE INFLUENCE OF OXYTOCIN ON ADIPOSE TISSUE, INFLAMMATION AND
ATHEROSCLEROSIS

By

Maria Agustina Rossetti

A THESIS

Submitted to the Faculty
of the University of Miami
in partial fulfillment of the requirements for
the degree of Master of Science

Coral Gables, Florida

December 2011

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THE INFLUENCE OF OXYTOCIN ON ADIPOSE TISSUE, INFLAMMATION AND
ATHEROSCLEROSIS

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The Influence of Oxytocin on Adipose
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Purpose: The present study investigates the potential anti-inflammatory effects of *in vivo* oxytocin (OT) infusion on adipose tissue inflammation in the Watanabe Heritable Hyperlipidemic Rabbits (WHHL).

Methods: Twenty-eight 3-month-old WHHL were surgically implanted with osmotic minipumps containing OT (n = 14, infusion rate 250 ng/kg/hr) or vehicle (n = 14). Blood samples were taken at baseline, midpoint, and endpoint for lipids and C-reactive protein (CRP). After 16 weeks, animals were sacrificed and samples of adipose tissue (epididymal, retroperitoneal, mesenteric, pericardial, and subcutaneous) were collected and analyzed for pro-inflammatory cytokine (IL-6, TNF- α , and MCP-1) and anti-inflammatory adipokine (adiponectin and IL-10) expression levels by Real Time-Polymerase Chain Reaction. Adipose tissue was also immunohistologically analyzed for macrophage infiltration. Aortas were dissected, formalin-fixed, and stained with oil-red O for en face quantification of lesion area. Student's t-tests were used to compare group means for all measures.

Results: Endpoint OT levels were significantly different ($p < .05$) between the control (M = 11.28 pg/ml, SEM = 2.5) and treatment group (M = 132.35 pg/ml, SEM = 8.5).

Plasma lipids were not altered by OT infusion. OT-treatment significantly decreased plasma CRP, a marker of systemic inflammation, at midpoint and endpoint compared to

controls ($p = 0.05$). OT-treated animals displayed significantly less atherosclerosis in the thoracic aorta ($p < 0.05$); a finding similar to our previously published study in a mouse model of atherosclerosis. In some fat depots, there was a trend suggesting adiponectin gene expression increased in the OT-treatment group. There were no significant differences or trends regarding macrophage infiltration in adipose tissue.

Conclusions: Oxytocin infusion attenuated thoracic aortic atherosclerosis, plasma CRP, and may affect inflammatory cytokine expression in adipose tissue in the WHHL model.

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Chapter 1: Overview

Increased adiposity is an important risk factor for a variety of diseases. Obesity, characterized by chronic low-grade inflammation of adipose tissue (Engstrom et al., 2003), predisposes individuals to a myriad of diseases associated with the metabolic syndrome such as insulin resistance, diabetes, (Gauthier and Ruderman, 2010) and cardiovascular diseases (Hubert et al., 1983). Obesity is typically accompanied by proliferation of adipose tissue (Coppack, 2005), which consists of adipocytes, macrophages, fibroblasts, blood cells, mesenchymal cells, pre-adipocytes, and endothelial cells (Fruhbeck, 2008). Although it is well established that adipose tissue functions as storage for excess energy, it has recently been recognized as a multifunctional organ that secretes over 100 factors that act in an endocrine/paracrine fashion (Hauner, 2004). These factors, termed adipokines, include pro and anti-inflammatory cytokines, growth factors, and proteins involved in vascular homeostasis, glucose metabolism, and angiogenesis (Trayhurn & Wood, 2004). Therefore there is increasing interest in adipose tissue as a dynamic endocrine organ and the role it plays in metabolic disorders and inflammatory diseases.

The pathophysiologic processes underlying cardiovascular disease (CVD), atherosclerosis, once believed to be a disease of lipoprotein accumulation, is now fundamentally understood as a chronic inflammatory disorder (Mullenix, Andersen, & Starnes, 2005). Systemic and local inflammatory events, driven largely by pro-inflammatory cytokines and oxidative stress, mediate all phases of atherogenesis (Ross, 1999). In our laboratory, we have demonstrated that social environment and emotional stress influences inflammatory and vascular oxidative stress (Nation et al., 2008) and

shed light on the effects of social experience in the progression of atherosclerosis in an animal model of disease, the Watanabe Heritable Hyperlipidemic (WHHL) (McCabe et al., 2002; Paredes et al., 2006). Rabbits subjected to a stable social experience, paired with a sibling, exhibited less aortic atherosclerotic lesions than rabbits subjected to an unstable social experience (paired with an unfamiliar male rabbit) and an isolation group where the rabbits were caged alone. Interestingly, animals that were individually caged and behaviorally sedentary gained more body weight and developed significant atherosclerosis (McCabe et al, 2002). Furthermore, the stable group exhibited significantly more affiliative social behavior and less agonistic behavior than the unstable group.

In recent years it has been established that the neuropeptide, oxytocin, modulates affiliative and prosocial behaviors (Ross & Young, 2009). The hormone also attenuates peripheral stress responses such as reducing cortisol levels and blood pressure (Moberg & Petersson, 2005). Oxytocin has also been shown to play a role in the cardiovascular system, working in the maintenance of normal homeostatic mechanisms. OT produced in the heart exhibits functions that may include slowing heart, decreasing contractility, and decreasing the mean arterial pressure via the local release of atrial natriuretic peptide, ANP (Gutkowska et al., 2000). Szeto et al., 2008 has demonstrated that OT may have antiatherogenic properties by inhibiting oxidative stress and inflammation in cultured endothelial vascular cells, monocytes, and macrophages. In vivo chronic OT infusions in Apo-E knockout mice attenuated aortic atherosclerosis and inhibited the secretion of pro-inflammatory cytokines, IL-6 in visceral adipose tissue (Nation et al., 2010).

Given these findings, the goal of the current study is to examine the potential role of adipose tissue in inflammatory responses that may contribute to the development of atherosclerosis. More specifically, levels of pro and anti-inflammatory adipokines (IL-6, IL-10, adiponectin, and TNF-alpha) expression was measured via RT-PCR in *ex vivo* adipose tissue following *in vivo* chronic OT infusion in WHHL rabbits. It is hypothesized that chronically elevated OT will directly suppress the expression of pro-inflammatory adipokines (IL-6 and TNF-alpha), and will promote the release of anti-inflammatory adipokines (adiponectin and IL-10). The effect of OT on macrophage infiltration will also be examined in adipose tissue through the use of immunohistochemistry. It is hypothesized that oxytocin will inhibit the infiltration of macrophages by inhibiting cell thickening of the fat tissue, providing another mechanism by which the hormone may attenuate the inflammatory process of adipose tissue. These experiments will shed light on the anti-inflammatory mechanism of OT, and its potential role in the attenuation of atherosclerosis.

Chapter 2: Introduction

Adipose Tissue

Storing excess energy in the form of triglycerides and organ protection from mechanical damage were believed to be the sole functions of adipose tissue (AT) for decades (Hauner, 2004). These firmly held beliefs coupled with disinterest deterred scientists from discovering the pleiotropic nature of the tissue. Discovering the multifaceted role of adipocytes, identifying obesity as a major health problem in society, and unveiling the dynamic role of adipose tissue as a complex and ubiquitous endocrine organ has enabled adipose tissue to be at the center stage of the research world (Fruhbeck, 2008).

The structure of adipose tissue, a type of loose connective tissue, is composed of adipocytes and a stroma-vascular fraction. Adipocytes make up anywhere from 25% to 70% of adipose tissue mass in adults. The remaining 30% is made up of other cells that make up the stroma-vascular fraction. Cells in the fraction include macrophages, fibroblasts, blood cells, mesenchymal cells, preadipocytes, small fat cells, and endothelial cells. Adipocytes range in size from 20 μ m to 200 μ m in diameter. In an adipocyte, the lipid droplet contains a mixture of cholesterol, phospholipids, triglycerides, fatty acids, and neutral fats. Triglycerides account for 95% of the contents of the lipid droplet. The lipids within the vacuole are continuously being removed and mobilized. These cells are capable of expanding their diameter twenty-fold and increase their volume several thousand-fold in order to accommodate excess lipid storage. Considering that 90% of an adipocyte is accounted for by the lipid droplet, the nucleus appears as a semi-lunar flattened structure pushed up on the side of the cell wall. In turn the cytoplasm

becomes a narrow sheath that is stretched around the lipid droplet. Organelles that can be seen in an adipocyte include a few mitochondria around the nucleus, a Golgi zone with free ribosomes, and a limited number of endoplasmic reticula with some lysosomes.

Adipose tissue refers to the sum of all depots excluding the fat found in the hands, feet, head, and bone marrow (Fruhbeck, 2008). It can be further subcategorized into internal adipose tissue and subcutaneous adipose tissue (Shen et al., 2003). Considering the endocrine function and depot-specific biological functions of adipose tissue, a classification system based on anatomical features is ineffective in identifying the different depots in the body (Shen et al., 2003). A review done by Shen et al., (2003) looked at over 100 articles that talked about imaging of adipose tissue compartments and how they relate to metabolic activity and disease. They reviewed articles from 1979 to 2002 that included the terms “total”, “regional”, and “visceral” adipose tissue. The review led them to create a total-body and regional adipose tissue classification. As mentioned above, the two umbrella categories of total adipose tissue mass are subcutaneous and internal adipose tissue. Subcutaneous tissue is divided into superficial and deep categories, and it makes up approximately 80% of the total adipose tissue mass. It is found in the lower trunk, mammary glands, and the gluteal thigh region. Superficial subcutaneous tissue is the tissue found between the skin and fascial plane (deep and fibrous connective tissue that surrounds the muscles) of the muscles. Deep subcutaneous tissue is the tissue found between the muscle fascia and the fascial plane of the regions mentioned above. Internal adipose tissue, which comprises approximately 20% of the total adipose mass, is categorized into visceral adipose tissue and non-visceral adipose tissue. Visceral tissue refers to the tissue within the chest, abdomen, and pelvis. Non-

visceral adipose tissue includes intramuscular and perimuscular (adipose tissue inside the muscle fascia).

Adipose Tissue and Inflammation

The new concept of adipose tissue function describes it as multifunctional organ that secretes over 100 factors such as proteins that act in an endocrine or auto/paracrine fashion (Hauner, 2004). The discovery of the adipocyte derived hormone leptin revolutionized research about adipose tissue and established it as an endocrine organ (Waki & Tontoz, 2007). Leptin acts on the central nervous system to regulate body weight by means of increasing the body's metabolic rate and restricting calorie intake (Rahmouni & Haynes, 2004). Soon after the discovery of leptin, the number of proteins secreted by adipocytes rapidly increased, and the term adipokine was established to give them a collective name (Trayhurn & Wood, 2004). The term is restricted to those factors released from the adipocytes and not adipose tissue in general, which also includes such as macrophages that also release proteins such as cytokines. Trayhurn & Wood (2004), summarize the wide range of adipokines. They include classical pro-inflammatory cytokines (e.g., IL-6 and TNF- α), growth factors (e.g., transforming growth factor- β ; TGF- β), proteins involved in vascular homeostasis (e.g., plasminogen activator inhibitor-1; PAI-1), glucose metabolism (e.g., adiponectin and resistin), and angiogenesis (e.g., vascular endothelial growth factor, VEGF). The wide range of protein signals and factors released from adipose tissue identify it as a dynamic endocrine organ that is involved in the role of metabolic control and the development of metabolic disorders.

Engstrom et al., (2003) describes obesity as disease of chronic low grade inflammation. Chronic inflammation of obese adipose tissue is characterized by elevated

levels of inflammation markers such as pro-inflammatory cytokines, IL-6 and TNF- α (Trayhurn & Wood, 2004). There is growing evidence that the chronic low grade inflammation state results in the development of insulin resistance, metabolic syndrome, and cardiovascular disease (Wellen & Hotamisligil, 2003). One of the theories postulated for the origin of the inflammatory markers states that the elevated production of markers is positively correlated to the increased adipose tissue mass (Trayhurn & Wood, 2004). A non-adipocyte fraction of adipose tissue, macrophages, may also play a significant role as their numbers increase in obesity and they also secrete factors that further perpetuate the inflammatory response (Weisberg et al., 2003).

TNF- α is the most studied adipocyte-derived cytokine (Coppack, 2001). TNF- α plays a major role in adipose tissue as it causes both systemic and local catabolic changes which in turn affect major metabolic pathways (Hauner, 2004). Coppack (2001) describes the effect of TNF- α on the development of insulin resistance, due to its inhibition of the insulin receptor signaling pathway. He also describes the powerful role that the cytokine plays within adipose tissue, inducing processes like apoptosis. In relation to other cytokines, TNF- α plays an important role in the production of other cytokines, adipokines, and inflammation markers. It exerts a stimulatory effect in the synthesis of IL-6, leptin and CRP, perpetuating the chronic low-grade inflammation observed in obesity and its comorbidities (Bullo et al, 2004)

Another adipocyte-derived pro-inflammatory cytokine of interest is IL-6. This cytokine is also involved in the regulation of metabolism (Hauner, 2004). One of the distinguishing factors of IL-6 is its release into systemic circulation. Approximately one third of the plasma IL-6 level is credited to adipose tissue production. Plasma levels and

expression in tissue of IL-6 have been shown to be elevated in obesity and insulin resistance. Considering the presence of IL-6 receptors in the hypothalamus, the cytokine may play a pivotal role in conveying information to the central nervous system regarding energy balance regulation (Mohamed-Ali et al., 1997).

An important adipokine known to be involved in obesity and insulin resistance is monocyte chemoattractant protein-1, or MCP-1. This adipokine promotes adipose tissue inflammation by recruiting circulating monocytes to migrate to the adipose tissue and become activated macrophages (Sell & Eckel, 2007). Monocyte migration from the blood stream is a part of immunological surveillance and the inflammatory response (Deshmane, Krenlev, Amini, & Sawaya, 2009). MCP-1 has also been linked to cardiovascular disease, playing a substantial role in arterial lipid deposition in atherosclerosis (Boring, Gosling, Cleary, & Charo, 1998).

IL-10 is an anti-inflammatory cytokine secreted by adipocytes (Trayhurn & Wood, 2004). In humans, IL-10 has been shown to attenuate the inflammatory response by suppressing the production of pro-inflammatory cytokines such as the previously described IL-6 and TNF- α (Juge-Aubry et al., 2004). IL-10 plays an important protective role that counteracts the effects of inflammation in obesity. This is evident in a study conducted by Esposito et al., (2003) that showed low serum concentrations of IL-10 are associated with metabolic syndrome in obese women.

Another important anti-inflammatory adipokine is adiponectin. Adiponectin is involved in glucose metabolism (Trayhurn & Wood, 2004). It is an abundant plasma protein that has a negative correlation with overall fat mass, increasing with weight loss and decreasing with weight gain (Hu et al., 1996). Adiponectin improves insulin

sensitivity (Galic, Oakhill, & Steinberg, 2009), and is positively related with glucose use and cardiovascular protection (Wang, & Scherer, 2008).

Growing evidence suggests that adipose tissue macrophages (ATMs) are the primary source of inflammatory mediators such as TNF- α and IL-6, and that these ATM derived cytokines interfere with proper function of adipocytes leading to insulin resistance and eventually ending in the development of metabolic syndrome (Zeyda & Stulnig, 2007). Weisberg et al., (2003) has shown that obesity-related adipose tissue inflammation is characterized by an increase in ATMs, evidenced by increased macrophage gene expression in AT and also immunohistochemical identification of ATMs. The increased number of ATMs results from the migration of circulating monocytes that differentiate into macrophages at sites of inflammatory responses (van Furth, Diesselhoff-den Dulk & Mattie, 1973). These monocytes undergo classical M1 activation induced by type 1 T-helper cell cytokines, for example, interferon-gamma (Mosser & Edwards, 2008). M1 activated macrophages results in an increase of pro-inflammatory cytokines at the migration site (Odegaard & Chawla, 2008). Cinti et al., (2005) has described the recruitment of phagocytes in obesity by increased necrosis-like adipocyte cell death in rodent obesity due to the damaging effects of cell hypertrophy. Dead adipocytes in obese rodents were surrounded by a crown structure formed by ATMs. Also noted in humans, but in low frequencies, obese individuals exhibit these crown-like ATM formations around necrotic adipose tissue.

Cardiovascular Disease and Adipose Tissue

Independent common vascular risk factors that are associated with adipose tissue dysfunction include elevated blood pressure, low plasma high-density lipoprotein

cholesterol (HDL), and high levels of triglycerides. These factors are associated with visceral obesity and can be reversed or controlled with weight loss and dietary restrictions. Signaling pathways within adipose tissue links the prevalence of cardiovascular disease associated with obesity. Obesity results in the modification of adipocyte structure leading to physiological dysfunction, characterized by changes in production and secretion of adipokines. These factors interact with other cells and tissues in the body, including multiple pathways that are linked to cardiovascular disease. Altered adipokines concentration mediate functions that are associated with cardiovascular disease such as fibrinolysis, coagulation, blood pressure, inflammation, insulin resistance, and the development of atherosclerosis (DeClercq, Taylor, & Zahradka, 2008; Hajer, Haeften, & Visseren 2008).

In regards to vascular disease, visceral and subcutaneous adipose tissues differ in their function. Visceral adipose tissue expresses higher levels of angiotensinogen (blood pressure regulation) and fatty acid-binding protein 4 (aids in fatty acid trapping in adipocytes) than subcutaneous fat (Dusserre, Moulin, & Vidal, 2000). One of the main adipokines, leptin, is produced mainly in subcutaneous tissue, while TNF- α is approximately equally produced in both fat depots. Also, macrophage infiltration is lower in subcutaneous adipose tissue (Cancello et al., 2006). Visceral adipose tissue also secretes higher levels of IL-6 and adiponectin (Hajer, Haeften, & Visseren 2008).

Regarding atherosclerosis, elevated plasma concentrations of adiponectin exert a protective effect on arteries. Studies reveal that adiponectin in plasma attaches to injured arteries (Okamoto et al., 2000). Also, elevated plasma adiponectin levels suppress the development of atherosclerotic lesions in Apo-E knockout mice (Okamoto et al., 2002).

Increasing serum adiponectin levels results in an inhibition of TNF- α induced endothelial adhesion molecules, in TNF- α expression in macrophages and adipose tissue, in inhibition of foam cell formation, and smooth muscle cell proliferation (Wu & Zhao, 2006). TNF- α and IL-6 are proinflammatory cytokines that act as hormones on vascular cells and promote the aggregation of lipids, contributing to the formation of atherosclerotic plaque (Ross, 1993).

Social Environment and Atherosclerosis

There has been extensive research done to support the idea that social environment can affect the development and progression of atherosclerosis (McCabe et al., 2002 & Kaplan, Chen, & Mancuk, 2009). More specifically, the experimental disruption of social environments has shown to stimulate atherosclerotic changes in the aorta and coronary arteries in a number of animal models (Mancuk, Kaplan, & Matthews, 1986). The effects of stressful psychosocial environment on atherosclerosis was first demonstrated by Ratcliff and Cornhill in 1958, when they reported that during a period of crowding at the Philadelphia Zoological Garden, due to increased animal populations, the incidence of atherosclerosis rose due to the stressful social pressure that came along with the condition.

A study conducted to shed light on the effects of positive social environment on diet-induced atherosclerosis found that rabbits that were exposed to individual petting, holding, and playing had 60% reduction of atherosclerotic regions compared to the control group (Nemere, Levesque, & Cornhill, 1980). Similarly, in a study conducted with swine, animals fed a high-cholesterol diet and raised in groups developed less atherosclerotic lesions than those raised alone or in pairs and fed the same diet. The

separation of the pigs after the group social bonds had been established led to the stressful situation that aided the progression of the lesions (Ratcliffe, Luginbuhl, & Chacko, 1969). A study using WHHL rabbits as the animal model further supported that social environment can attenuate (positive social environment) as well as accelerate (negative social environment) the progression of atherosclerosis (McCabe et al., 2002). In the mouse model of atherosclerosis, Apo-E mice, findings suggest that isolated housing leads to increased atherosclerotic disease compared to mice that were housed in groups of twelve (Bernberg & Andersson et al., 2008).

Regarding non-human primates, it has also been shown that cynomolgus monkeys housed in unstable social conditions exhibited had significantly greater coronary artery atherosclerosis than monkeys in stable social conditions (Kaplan, Manuck, Clarkson, Lusso, & Taub, 1982). In humans, interpersonal support has been shown to decrease heart rate reactivity while performing laboratory tasks in a study conducted by Kamarck, Manuck, and Jennings (1990). Heart rate in humans has also been shown to decrease during non-self tactile stimulation (Drescher, Horsley Gantt, & Whotehead, 1980), further indicating the benefits of positive social interactions. A study that examined personality and social predictors of atherosclerotic progression found that social deprivation directly affected atherosclerotic progression as measured by the noninvasive ankle brachial pressure index (ABPI). Low ABPI indicates arterial disease. Greater social deprivation predicted lower ABPI scores for men in the study (Whiteman, Deary, & Fowkes, 2000).

Oxytocin, Social Behavior, and Stress

Oxytocin is a neurohypophyseal hormone commonly known to play a part in mammalian parturition and milk ejection (Carter, Williams, Witt, & Insel, 1992). However, it has recently been shown that the hormone is involved in pro-social affiliative behavior and in the central nervous system's neuroendocrine response to stress (Heinrichs, Bauggartner, Kirschbaum, & Ehlert, 2003). Pro-social behaviors are denoted by repeated physical contact, for example touch, exchange of warmth and olfactory cues (Uvnas-Moberg & Petersson, 2005). OT has been shown to be the driving force behind such behaviors as nest-building, licking the pups, and crouch-posture in rats (Argiolas & Gessa, 1990). In prairie voles, oxytocin has been proven to increase pro-social behavior, such as social bonds, and also reduce stress related hypothalamic-pituitary-axis (HPA) activity (Carter, 1998). OT has emerged as regulatory mediator in the context of pro-social behaviors. Through an increase in parasympathetic vagal nerve activity, OT has also been shown to increase anabolism, induce anti-anxiolytic effects, and promote healing and growth (Uvnas-Moberg & Petersson, 2005). Similarly, the anti-stress pattern generated by the OT is characterized by decreased cortisol levels, lowered blood pressure, increased weight-gain, and an increased release of gastrointestinal hormones (Uvnas-Moberg, 1998).

Oxytocin, Inflammation and Atherosclerosis

The role of OT in the heart includes slowing of the heart (Argiolas & Gessa, 1990), decreasing contractility, and decreasing the mean arterial pressure via the local release of atrial natriuretic peptide (ANP) (Gutkowska et al., 2000). A potential mechanism by which oxytocin may slow the development of atherosclerosis is postulated

in an experiment done with rats suggests that identified oxytocin receptors in the endothelial smooth muscle cells of the heart and aorta may regulate vascular tone and growth (Jankowski et al., 1998; Jankowski et al., 2000).

Most of the work done linking oxytocin, inflammation and atherosclerosis has been conducted at our lab where Szeto et al., (2008) found that OT attenuated the production of NADPH-dependent superoxide and the release of pro-inflammatory cytokine, IL-6, from *in vitro* macrophages. These findings suggest that oxytocin could potentially play a significant role in slowing down the progression of atherosclerosis. To further expand on these results, Nation et al., (2010), showed that OT attenuated atherosclerotic lesion development as well as adipose tissue inflammation in socially isolated Apo-E mice. These findings further promote the idea that OT and affiliative behavior have a potential significant role in the attenuation of disease.

Chapter 3: Hypotheses

1. Chronically elevated plasma OT levels will directly suppress the expression of adipokines shown to promote all phases of atherosclerosis.
 - a. Oxytocin will inhibit the production of pro-inflammatory adipokines that are important in the pathophysiology of atherosclerosis: IL-6, MCP-1, and TNF- α .
2. Chronically elevated plasma OT levels will promote the production of anti-inflammatory adipokines that are important in the attenuation of disease.
 - a. OT will upregulate the gene expression of adiponectin and IL-10
3. Oxytocin will affect the infiltration of macrophages to the adipose tissue.
 - a. OT will decrease or inhibit the infiltration of circulating monocytes and macrophages to adipose tissue.

Chapter 4: Methods

The current study was part of a larger experiment that examined the effects of elevated oxytocin plasma levels on the progression of atherosclerosis in the WHHL rabbit. The methods for this larger study and the current study are presented below.

Experimental Animals

Twenty-eight WHHL rabbits, 3 months of age and weighing 2.0-3.5kg, were purchased from Brown Family Enterprises, LLC. Rabbits were randomized to control (n=14) and experimental (n=14) groups on arrival and were individually housed in a temperature- and humidity-controlled environment on a reverse 12hr light/dark cycle and given food and water *ad libitum*. Rabbits were acclimated for 7 days before initiation of experiments. All experimental procedures were approved by the Institutional Animal Care and Use Committee of the University of Miami.

Surgeries

Primed (70hrs) osmotic mini-pumps that would deliver 250 ng/kg/hr OT or vehicle (VH), 50mM Sodium Citrate, pH = 4, were surgically implanted subcutaneously. Pump-exchange surgeries performed every 4 weeks for three months were also identical except for the removal of previously implanted pumps. After surgical removal, pumps were stored at 4°C until analyses could be performed to confirm OT stability and pump function. Replacement of pumps was accomplished by deeply anesthetizing the rabbit with a gas mixture of 1-3% isoflourane in 100% oxygen and through the use of aseptic surgical procedures.

Tissue Collection

On the date of sacrifice (12 weeks after beginning OT infusion) rabbits were euthanized with Euthasol (~ 1 cc/3kg) into marginal ear vein. After sacrifice, the

epididymal, mesenteric, pericardial, subcutaneous, and retroperitoneal fat depots were weighed and collected in an RNA lysis buffer for gene expression analysis. Tissues were stored in 10% buffered formalin for later histological analysis and quantification of atherosclerotic disease.

Plasma OT Levels

To verify elevated plasma OT levels in the treatment group, plasma was extracted and assayed for OT with blood extracted at baseline, midpoint (at 8 weeks), and endpoint (at 16 weeks). Solid phase extraction of samples was performed using 200 mg C18 Sep-Pak columns (Bachem, San Carlos, CA). A commercially available enzyme-linked immunosorbent assay kit was used to measure extracted plasma for oxytocin (ADI-900-153; Enzo Life Sciences, Plymouth Meeting, PA).

Plasma CRP, Lipids, Biomarkers and Atherosclerotic Quantification

As part of the overarching experimental design, blood samples were also analyzed for C-reactive protein (CRP), a marker of systemic inflammation, lipids (cholesterol and triglycerides), and biomarkers (cortisol and oxidized-LDL). Aortas were dissected, formalin-fixed, and stained with oil-red O for *en face* quantification of lesion area.

RT-PCR analysis of adipokines

Total RNA (optical density ratio of 260/280 nm, >1.8) was isolated from approximately 50 mg of adipose tissue using the RNeasy kit (Qiagen, Valencia, CA) and treated with DNase I (Biosystems, Carlsbad, California). cDNA was prepared using the reverse transcriptase reaction (High-Capacity cDNA Reverse Transcription Kits #4374967, Applied Biosystems) following manufacturer's protocol. Primers for polymerase chain reactions (PCR) were from Applied Biosystems; IL-6 (cat

#Oc03822686_S1), MCP-1 (cat # Oc03823307_S1), adiponectin (cat # Oc03823307_S1), IL-10 (cat# Oc03396942_M1), TNF- α (cat# Oc03397715_M1) and 18S (cat# Hs03928990_G1).

Quantitative gene expression of the primers was performed with the use of real-time PCR and with TaqMan gene expression assay (#4352042, Applied Biosystems). Twenty micrograms of cDNA were amplified with TaqMan Universal PCR Master Mix and reactions run using universal cycling conditions on an Applied Biosystems Step-One Plus RT-PCR System. Samples were analyzed in triplicate and were normalized to the housekeeping gene, 18S. To analyze relative quantitation (RQ), the Comparative Ct method $\Delta\Delta$ CT (threshold cycle) method (Pfaffl, 2001) was used. RQs were used to analyze the difference in gene expression in the two groups: control and oxytocin infusion. The control for the calculations was the control group. The RQ results were expressed as the fold change in gene expression. This is explained in more details in the statistical analysis section.

Immunohistochemistry

For staining of macrophages in adipose tissue, fixed tissue was sliced into 10 micron sections and then immuno-stained by the avidin-biotin peroxidase (ABC) technique (Vector # PK-4002). Deparaffinized slides were pretreated with an antigen retrieval method intended to break the cross linkages formed by formalin fixation (Shi, Cote, & Taylor, 1997). Following antigen retrieval, the slides were processed in the following order: (1) hydrogen peroxide (3% final concentration) in tap water for 5 minutes to quench endogenous peroxidase activity; (2) normal horse serum (1:67) for 20 minutes to reduce background non-specific staining; (3) incubated with primary rabbit

macrophage antibody, monoclonal anti-mouse RAM-11 (DAKO # M063301-8), diluted 1:50 in PBS for 30 minutes at room temperature; (4) incubated with biotinylated secondary antibody provided in kit for 30 minutes at RT; (5) ABC complex for 30 minutes; (6) incubated sections with peroxidase substrate, Diaminobenzidine(DAB Vector # 4100) until desired stain intensity; (7) counterstained with hematoxylin(Vector # H-3404) and mounted with Histomount (Invotrogen # 00-8030). The appropriate isotype, IgG₁, was used as the control isotype.

Statistical Analyses

To quantitatively assess inflammation in the five adipose depots collected, (epididymal, retroperitoneal, pericardial, mesenteric, and subcutaneous) gene expression for IL-6, MCP-1, Adiponectin, IL-10, and TNF- α , was analyzed for both the control and oxytocin groups via the RT-PCR method. The comparative Ct method was used to analyze the data. The RT-PCR method calculates Ct (threshold cycle) values for the gene of interest. The Ct value is a relative measure that inversely proportional to the initial quantity of cDNA (Wang et al., 2007). Lower initial cDNA concentrations will require more PCR cycles (higher Cts) in order for the signal to reach the threshold line for detection (Wang et al., 2007). Thus, the Ct value for a given gene is equal to the interaction between an amplification curve and a threshold line (Applied Biosystems, Application Note, 2008). The next step of the analysis is the relative quantification, which in this case is done by normalizing the genes Cts to the reference gene, 18S. It is important the reference gene show constant values across experimental and control groups (Wang et al., 2007). In the present study, there were outlying cases in the 18S reference values of all depots except the mesenteric fat depot. The calculations of the

Comparative Ct method for analysis were conducted excluding the 18S outliers for each depot. Although the exclusion of outliers may lead to a small sample size and in turn to a loss of statistical power, the results are more representative and consistent across animals and across groups.

For the present study, 18S was selected to normalize and account for the inter-PCR variation by calculating the ΔCt value. This value is the difference between the target gene Ct and the Ct for the 18S housekeeping gene. The next step is to calculate the $\Delta\Delta Ct$, and this value represents specific gene expression under an experimental condition. The value is calculated by subtracting the ΔCt s of the target gene from the ΔCt s of the control samples. This value is then plugged into the following equation to determine the fold change in gene expression, $2^{-\Delta\Delta Ct}$, and the resulting value is the Relative Quantitation (RQ). The RQ equation accounts for the exponential amplification of the RT-PCR method where a $\Delta\Delta Ct$ of 1 equals a two-fold change in gene expression and a $\Delta\Delta Ct$ of 2 equals a 4 fold-change and so on (Wang et al., 2007). In the present study, to be able to perform statistical analyses the $\Delta\Delta Ct$ s and RQ were calculated two different ways. The first sets of calculations were group based and they were performed to analyze the fold-change for overall gene expression due to treatment, relative to the control group (Livak & Schmittgen, 2001, Schmittgen & Livak, 2008). In this case, the mean $\Delta\Delta Ct$ s and the mean RQs for each group was calculated and compared. The second set of calculations was individually based and they were used to perform the statistical test to look for group differences. For this set of analysis, the $\Delta\Delta Ct$ s and the RQs were analyzed for all the rabbits individually. An independent sample two-tailed Student's t-test was conducted with the individual RQs of each rabbit to examine group differences.

Analysis of histological data was conducted by capturing 64 images of the stained tissue and creating a visual field where to count the macrophages (40X magnification, QCapturePro program and GImaging camera for a Leica DM 1000). The images were stitched together to form one image with the help of Microsoft Image Composite editor. With Metamorph, a grid of 7 by 5 equally spaced squares was overlaid over the final composite images. In excel, a random number generating equation will produce 12 random numbers from 1-35. These 12 numbers were assigned to the squares that were analyzed for macrophages. Following the cell count, the total number of macrophages of the 12 squares was normalized for area by dividing the total number of macrophages counted in the 12 squares by the area of one square in mm^2 . Means and SEMs were determined for the vehicle and the treatment group and an independent sample Student's t-test was conducted to determine group differences.

Chapter 5: Results

Plasma OT Values

Oxytocin levels in control and oxytocin-treated WHHL rabbits for baseline, midpoint, and endpoint are displayed in Figure 1. OT levels were significantly different at all time points with a p-value of 0.001.

Plasma CRP, Lipids, Biomarkers and Atherosclerotic Quantification

OT-treatment significantly decreased plasma CRP at midpoint and endpoint compared to controls ($p = 0.05$), as displayed in Figure 2. Weights, plasma lipids (Table 1) and biomarkers (Table2) were not altered by OT infusion. Figure 3 shows the OT-treated animals displayed significantly less atherosclerosis in the thoracic aorta ($p < 0.05$); a finding similar to our previously published study in a mouse model of atherosclerosis (Nation et al., 2010).

RT-PCR Results

When analyzing RT-PCR data, the first step is to internally account for error, and this is done by normalizing gene expression data to an endogenous gene (Hugget, Dheda, Bustin, & Zumla, 2005). For this experiment, data was normalized to the housekeeping gene, 18S. Potential problems present themselves when animals do not express appropriate endogenous gene (18S) levels. In the present study, certain animals did not express acceptable levels of the housekeeping gene even when repeating the samples. Due to this reason, outliers were determined by analyzing the 18S levels of all adipose depots. Ct values of the endogenous genes affect the proceeding calculations of the Comparative Ct method for the analysis of PCR data, thus extreme 18S levels will affect the analysis and interpretations of the results. A method for identifying potential outliers

is to display the data in a box-plot graph (Burns, Nixon, Foy, & Harris, 2005). The box-plot graph identifies outliers as values that lay beyond the 5% to 95% range that is represented by the whiskers of the box. Figure 4 displays the boxplots for all depots with sample outliers designated by an asterisk. It should be noted that the numbers on the box-plot represent the case number and not the animal number. Outliers were identified in all fat depots, except the mesenteric depot. The following animals were excluded for the analysis of the RT-PCR data for the different adipose depots (O-Oxytocin group and C-control group): epididymal (animal # 26-C), retroperitoneal (animals # 5-C, 16-C, 18-O, and 26-C), subcutaneous (animal #15-O), and pericardial (animals # 21-O, 23-O, 26-C, and 31-C).

Figure 5 displays group's fold change in gene expression due to treatment for the epididymal fat depot. Statistical analysis were conducted on the individual animal's relative quantitation number and no significant results were found for any of the adipokines (IL-6, MCP-1, Adiponectin, IL-10 and TNF- α). Although there were no statistically significant differences between the control and oxytocin groups, there was a noticeable trend. The difference in adiponectin gene expression was close to reaching significance with a p-value of 0.0632.

In Figure 6, group mean RQ results of all the adipokines for retroperitoneal adipose tissue are presented. The group relative quantitation, interpreted as the fold change in gene expression due to treatment, did not differ between groups for any of the genes. Individual quantitation used to perform the statistical analysis and there were no significant differences or trends seen in these results.

There were also no significant results and no trends for mesenteric fat (Figure 7) and pericardial fat (Figure 8). Finally, there were no statistically significant results for subcutaneous adipose tissue (Figure 9) for any of the adipokines. However, there was a trend for adiponectin, with rabbits in the treatment group expressing a 1.83 fold-change of adiponectin gene expression compared to the control rabbits. The p-value for the two tail t-test for this analysis is 0.127, and although not normally considered a trending p-value, in this case due to the small sample size of the groups, it should be seen as a point of interest.

Immunohistochemistry

Immunohistochemical sections of epididymal adipose tissue were analyzed for the presence of macrophage, quantified by the number of cells present per area square of tissue. Tissue sections were blocked, paraffanized, and later sliced into 10 μ m slides. Sections were stained with RAM-11 and counterstained with hematoxylin. There was no significant difference in the number of macrophages found in the epididymal adipose tissue of the treatment vs. control groups, p-value = 0.483 (Figure10). A representation of the stained macrophages can be seen in Figure 11.

Chapter 6: Discussion

The purpose of the present study is to investigate the effects of infused oxytocin on adipose tissue inflammation, in a rabbit model of atherosclerosis. We hypothesized that chronically elevated levels of oxytocin would directly suppress the adipose tissue expression of pro-inflammatory cytokines (TNF- α , IL-6, and MCP-1) that aid in the development and progression of atherosclerosis. Similarly, it was hypothesized that chronic OT infusion would promote the production of anti-inflammatory adipokines important in the attenuation of inflammation and disease (adiponectin and IL-10). The RT-PCR method was used to quantitatively assess inflammation and cytokine gene expression (IL-6, MCP-1, Adiponectin, IL-10, and TNF- α) in the five adipose depots collected (epididymal, retroperitoneal, pericardial, mesenteric, and subcutaneous). Histological methods were used to analyze macrophage infiltration in the most metabolically active adipose depot, epididymal. Although there were no significant differences between control and treatment rabbits, there are a few points of interest that should be discussed. For the present study, the dose of OT infused through the miniosmotic was five times higher than physiological levels. However, this may have not represented a sufficient dose for pharmacological effects. Higher doses of the peptide may be necessary for oxytocin to affect the inflammatory state of the WHHL rabbits and modulate gene expression of cytokines and macrophage infiltration. Furthermore, as evidence by the percent of disease in the aortas of both groups, the current OT dose is strong enough to affect the progression of atherosclerosis in the thoracic aorta. However, a higher dose may have a more widespread effect on aortic atherosclerosis

Sampling of the adipose tissue during tissue collection may have affected the results of the present study. An unpublished study from our lab has revealed that epididymal adipose tissue may not have uniform metabolic activity across its different segments. In that study, the proximal and distal (relative to the testes) segments of the epididymal fat pad were analyzed from high-fat fed mice and normal chow fed mice. Inflammatory cytokine secretion and distribution of macrophage cells within the two regions differed not only between the two groups but also within the high-fat fed mice. For the obese mice, gene expression for adiponectin was significantly higher in the distal portion of the epididymal pad versus the proximal portion ($p=0.004$; Figure 12). Furthermore, for the present study one of the five adipose tissue depots collected was the pericardial fat pad. Due its proximity to the atherosclerotic disease of the animals, one may assume that the metabolic activity of this pad would be elevated. However, pericardial fat is not the most adjacent adipose depot to the heart, and its blood supply is derived from non-coronary sources. Furthermore, the metabolic role of pericardial adipose tissue is partially unknown and it should not be confused with the metabolically active epicardial tissue that lies closest to the heart between the myocardium and the visceral pericardium (Iacobellis & Malavazos, 2010). Recent studies have shown an increase in pro-inflammatory cytokine gene expression and macrophage infiltration in epicardial fat in patients with coronary atherosclerotic disease (Hirata et al., 2011; Zhou et al., 2011). In future studies, more careful tissue sampling of metabolically active areas of the fat pads (i.e., distal epididymal fat and epicardial adipose tissue) will be crucial in examining group differences in cytokine gene expression and macrophage infiltration.

Adipokine gene expression is stimulated in the presence of chronic low-grade inflammation due to obesity. Although the rabbits in the present study had atherosclerosis, they were not obese and thus the gene expression of the adipose depots may not be elevated as indicative of disease and systemic inflammation. It is possible that the adipose tissue was already at a low state of inflammation and the OT had no ability to reduce the basal inflammatory state. However, there was a trend with the oxytocin animals exhibiting higher levels of gene expression for adiponectin than the control animals in both the epididymal and subcutaneous adipose depots. This suggests that OT may have an effect on the gene expression of adiponectin in adipose tissue, more specifically epididymal and subcutaneous adipose depots. Adiponectin has been shown to be a protective factor in the first stages and progression of atherosclerosis by regulating NO (Nitric Oxide) production of endothelial cells, inhibiting monocytes adhesion to endothelial cells, inhibiting the transformation of macrophages to foam cells, and inhibiting the migration and proliferation of smooth muscle cells (Shimada, Miyazaki, & Daisa, 2004). Several animal studies have shown the protective effects of adiponectin on atherosclerosis. In a study conducted with adiponectin deficient mice, the animals showed severe wall-thickening as well as an increase in endothelial cell proliferation in injured arteries (Matsuda et.al., 2002). Similarly, adiponectin knock-out mice showed a 2-fold increase in neointimal formation compared to control wild-type mice (Kubota, et. al., 2002). Moreover, catheter-injured rat arteries immunohistologically showed an accumulation of adiponectin protein compared to the non-injured arterial walls, providing a repair mechanism when the endothelial barrier is injured (Okamoto, et.al, 2000). In the rodent model of atherosclerosis, the Apo-E deficient mouse, adiponectin has been shown

to migrate to the foam cells and have a direct effect in inhibiting the progression of disease (Okamoto et al., 2002). Furthermore, visceral adipose tissue inflammation in obesity has been established to accelerate atherosclerosis in Apo-E deficient mice (Ohman et al., 2008), and as discussed before, obesity leads to a decrease in adiponectin mRNA levels (Lihn et al., 2004) and plasma concentration (Yang et al., 2001). In the present study, the WHHL rabbits were not obese, however, OT may provide a first line mechanism by which it promotes an increase in the adiponectin production that migrates to the injured arteries and slows down the progression of the disease. If the animals would have been obese, there might have been a sharper difference in adiponectin production in OT vs. control animals, with the OT animals up regulating gene expression of the protein at a more rapid rate. To complement and further investigate the trends in the study, it would be crucial to perform immunohistological studies of adiponectin in the fat depots to see if increased gene expression translates to increased protein (at this time there is no available rabbit adiponectin antibody). Furthermore, measuring plasma adiponectin would also be important in order to assess its systemic effect. Considering that adiponectin is made exclusively by adipocytes, it provides a clear starting point that would facilitate the study of its anti-inflammatory mechanisms.

Macrophage infiltration consists of circulating monocytes migrating and differentiating into macrophages at sites of inflammatory responses (Stein, Keshav, Harris, & Gordon, 1992). For the present study there were no group differences in the amount of tissue resident macrophages found in epididymal adipose tissue. This could be attributed to the non-obese and non-inflammatory state of the animal's fat. Obesity is characterized by chronic low-grade inflammation of adipose tissue (Engstrom et al.,

2003) and is typically accompanied by proliferation of adipose tissue (Coppack, 2001) cell components such adipocytes, macrophages, fibroblasts, blood cells, mesenchymal cells, pre-adipocytes, and endothelial cells (Fruhbeck, 2008). It has been shown that obesity leads to the recruitment and accumulation of resident ATMs. Furthermore, obesity induces a phenotypic switch in macrophages resulting in the propagation of the inflammatory response. Adipocytes also play an important role in inflammation, acting as a source of cytokines, adipokines, and regulating immune responses via cross-talks with lymphocytes. Together ATMs and adipocytes propagate the chronic inflammatory state of obesity (Fantuzzi, 2005). Studies have shown that in diet induced obese mice, ATM's showed a decreased expression of IL-10 and an increase in inflammatory macrophage factors, TNF-alpha and iNOS (Modolell et al., 1995; Lumeng, Bodzin, & Salitiel, 2007). Furthermore, in a human study of moderate weight loss in obese women revealed that weight loss led to a significant decrease in macrophage infiltration in white adipose tissue (Cancello & Clement, 2006). For the present study, there were no group differences for macrophage infiltration because the rabbits were not overweight and their adipose tissue was not in a state of low-grade chronic inflammation.

Finally, a large sample size would increase the statistical power of the study and elucidate group differences. With the help of the statistical program, G-power, the calculated sample size needed to have a large effect size of 0.8 at an α error probability of 0.05 for an independent sample t-test is 42 animals per group, with a total sample size of 84. Increasing our sample size should be considered for future studies. However, other matters including available housing, costs, and time-efficiency with procedures should also be taken into account when dealing with such large animal sample sizes.

Overall, the present findings suggest that OT treatment can reduce thoracic aortic atherosclerosis lesion and may, at higher doses, affect inflammation markers in adipose tissue.

Table 1. Mean Values of Body Weight, Serum Lipids Across Time

	Vehicle (n=11)	Oxytocin (n=11)
Weight (kg)		
Baseline	3.02 ± 0.09	2.87 ± 0.10
Midpoint	3.26 ± 0.07	3.16 ± 0.09
Endpoint	3.54 ± 0.07	3.45 ± 0.12
Cholesterol (mg/dl)		
Baseline	596.36 ± 53.18	586.45 ± 37.12
Midpoint	601.27 ± 43.20	621.18 ± 36.71
Endpoint	541.45 ± 44.30	535.82 ± 35.95
Triglycerides (mg/dl)		
Baseline	316.45 ± 28.65	313.82 ± 38.30
Midpoint	253.73 ± 44.22	211.73 ± 35.63
Endpoint	227.82 ± 36.47	195.82 ± 35.72

Values are mean ± standard error of the mean. Baseline is pretreatment. Midpoint is after 8 weeks of treatment. End point is after 16 weeks of treatment.

Table 2. Mean Values of Biomarkers Across Time

	Vehicle (n=11)	Oxytocin (n=11)
Cortisol ($\mu\text{g/dl}$)		
Baseline	0.15 \pm 0.11	0.16 \pm 0.05
Midpoint	0.11 \pm 0.03	0.36 \pm 0.17
Endpoint	0.20 \pm 0.04	0.18 \pm 0.05
Oxidized LDL (U/l)		
Baseline	33.27 \pm 1.38	32.41 \pm 1.31
Midpoint	33.29 \pm 1.80	30.32 \pm 1.78
Endpoint	27.24 \pm 1.40	27.24 \pm 1.11

Values are mean \pm standard error of the mean. Baseline is pretreatment. Midpoint is after 8 weeks of treatment. End point is after 16 weeks of treatment.

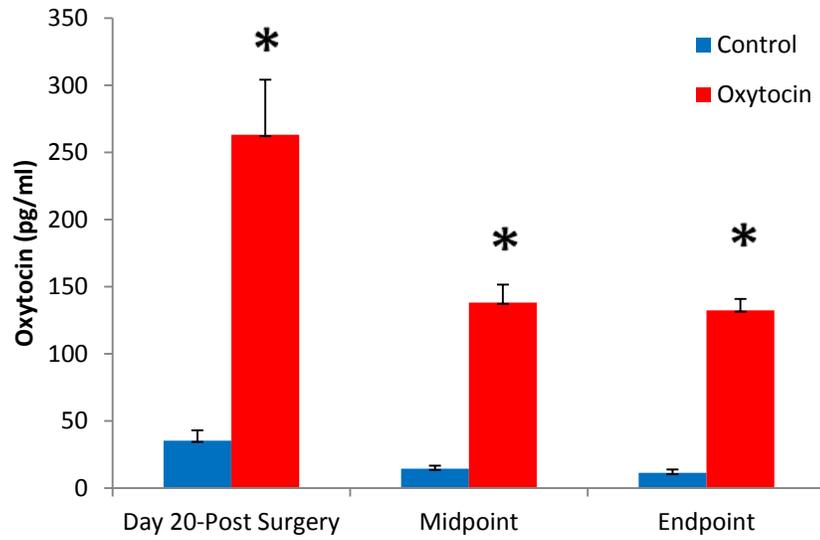


Figure 1. Oxytocin levels in control and oxytocin-treated WHHL rabbits.

* $p = 0.001$ between Groups

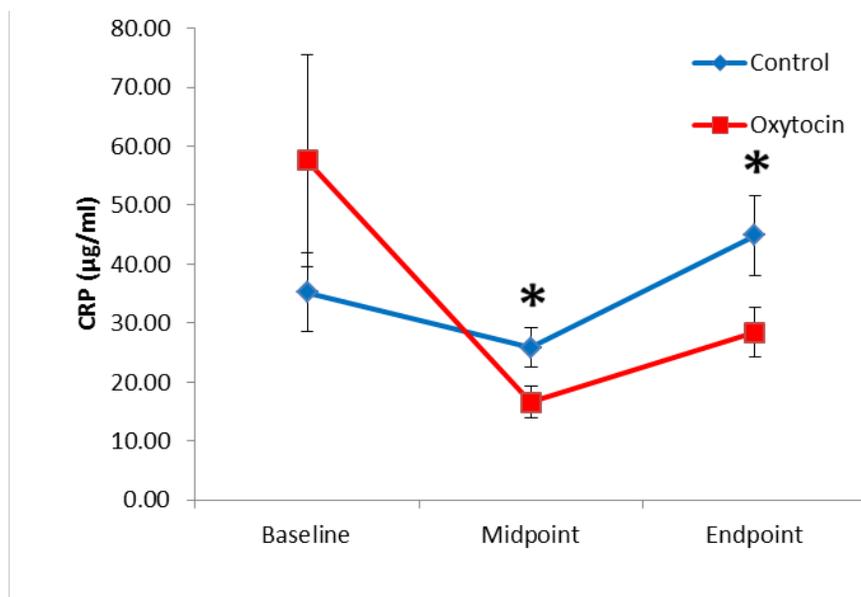


Figure 2. CRP levels in control and oxytocin-treated WHHL rabbits.

*p < 0.05 between Groups

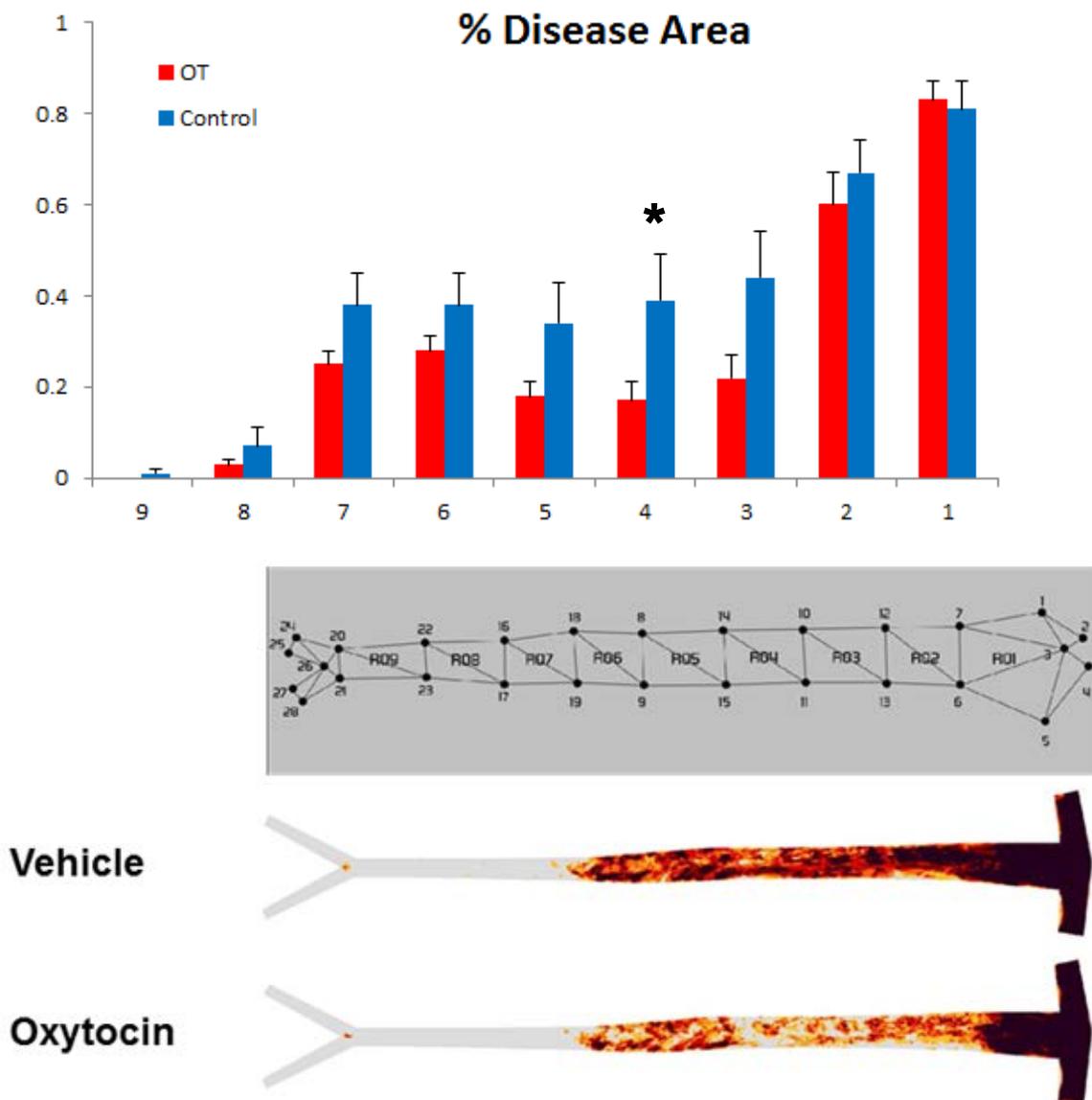


Figure 3. Extent of atherosclerosis in control and oxytocin-treated WHHL rabbits

*p = 0.049 between Groups

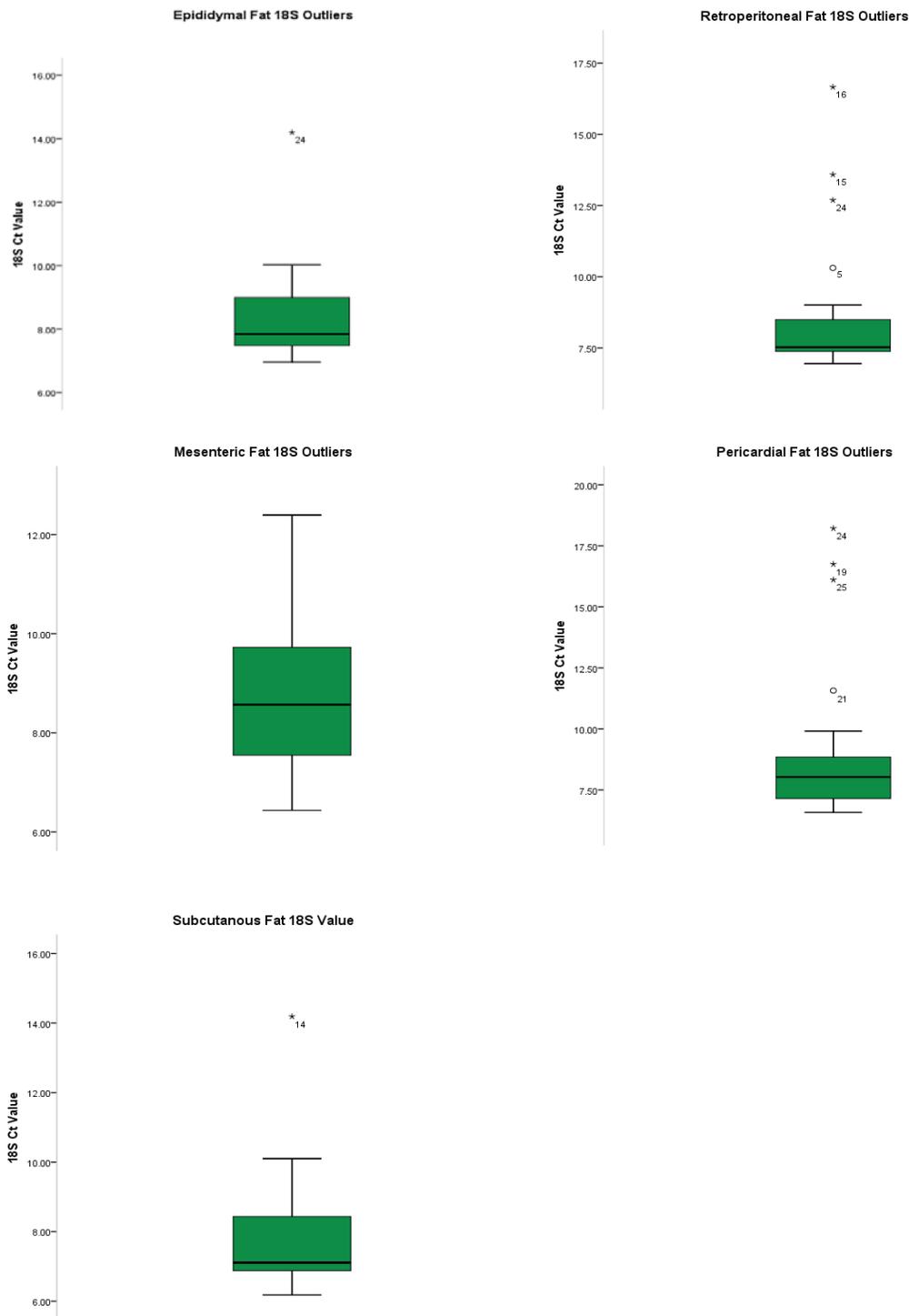


Figure 4. Endogenous gene, 18S, outliers for all fat depots. Outliers are case numbers and not anima numbers.

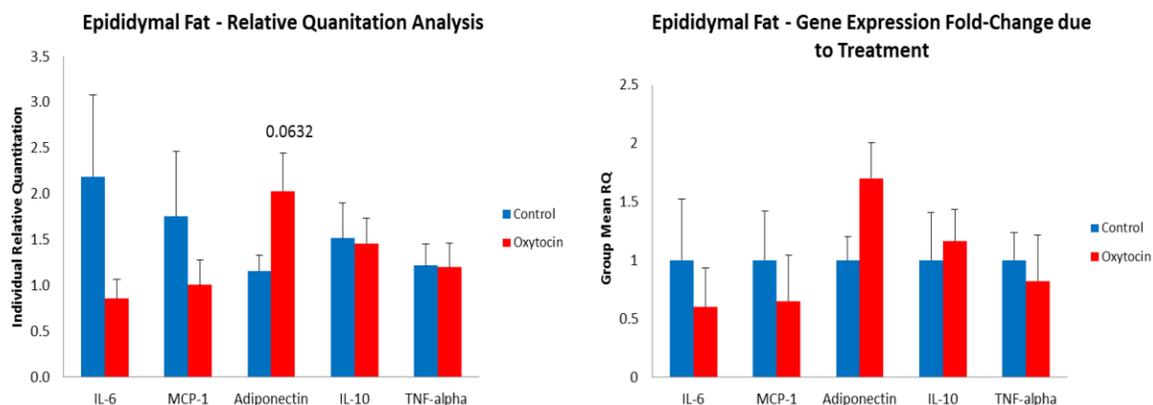


Figure 5. Relative quantitation analysis and gene expression fold- change for epididymal adipose tissue cytokine expression

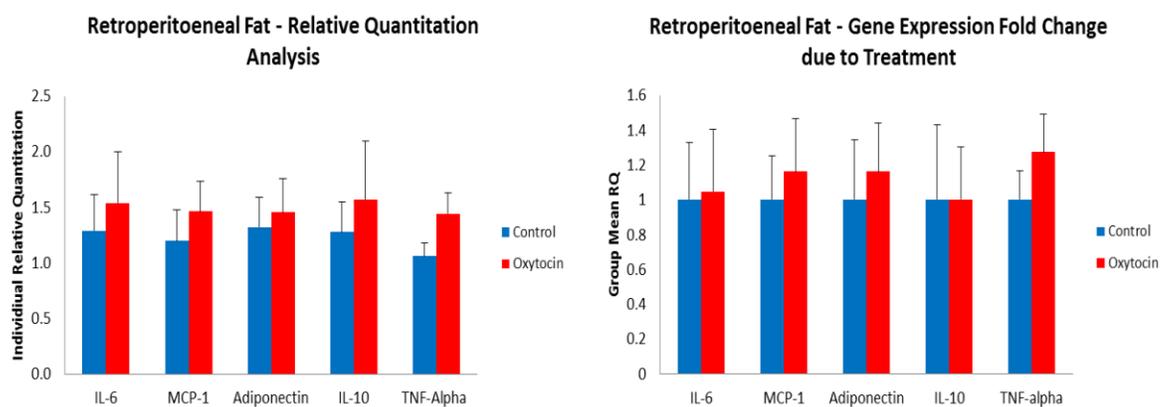


Figure 6. Relative quantitation analysis and gene expression fold- change for retroperitoneal adipose tissue cytokine expression

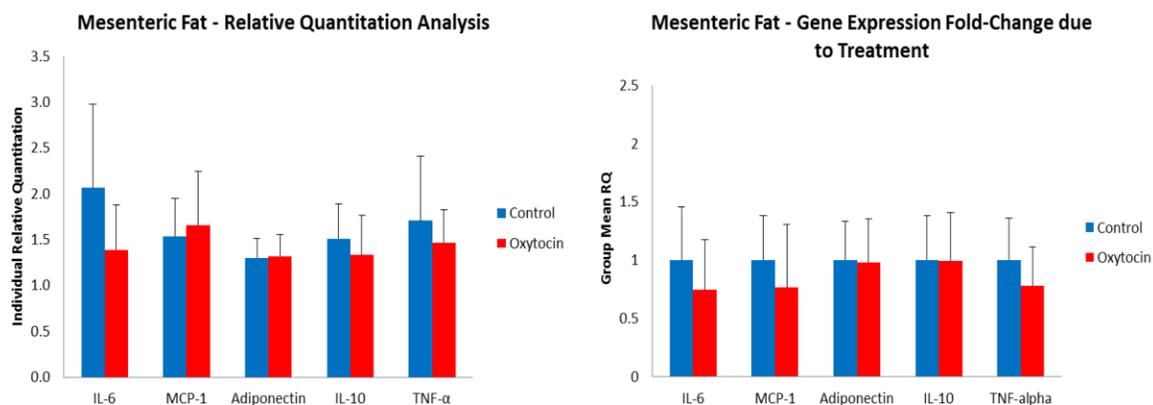


Figure 7. Relative quantitation analysis and gene expression fold- change for mesenteric adipose tissue cytokine expression

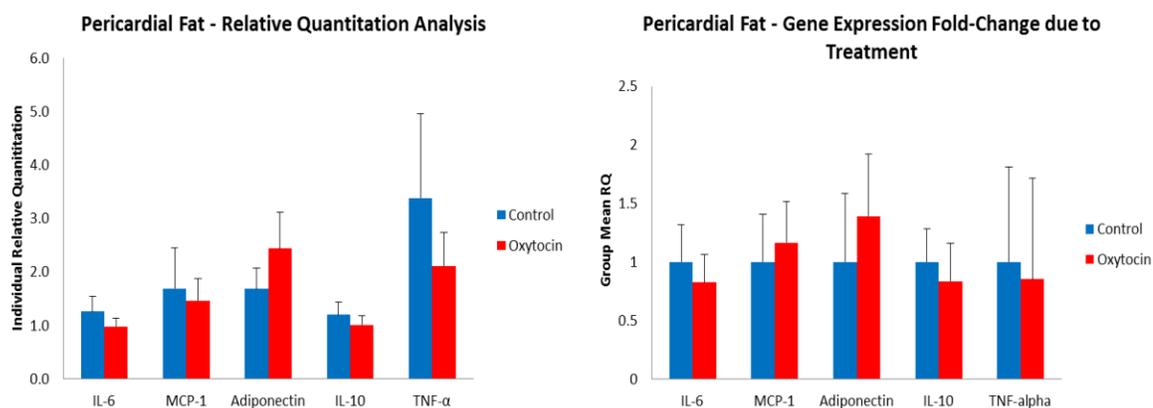


Figure 8. Relative quantitation analysis and gene expression fold- change for pericardial adipose tissue cytokine expression

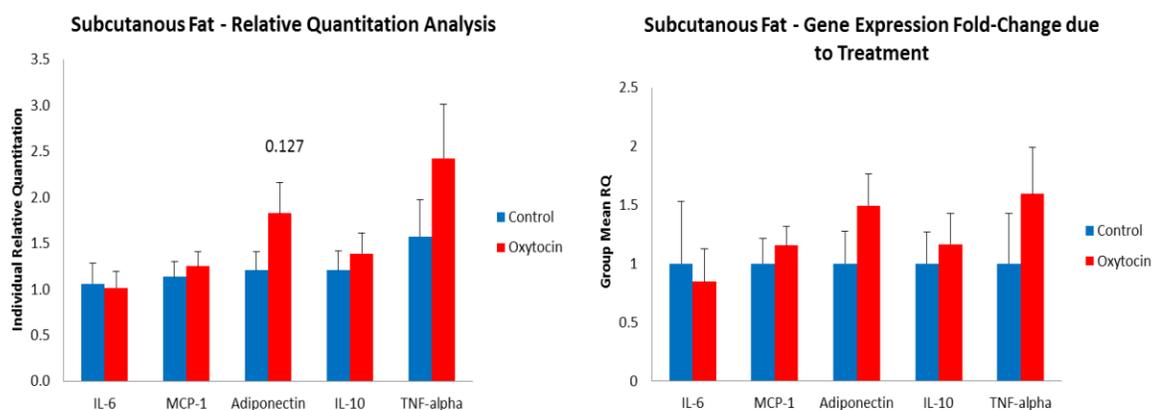


Figure 9. Relative quantitation analysis and gene expression fold- change for subcutaneous adipose tissue cytokine expression

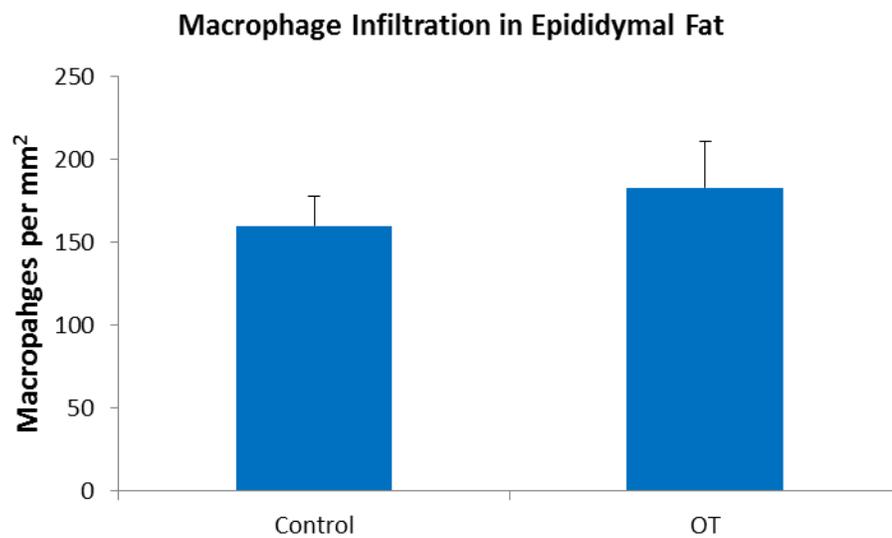


Figure 10. Histological quantification of macrophage infiltration in epididymal adipose tissue.

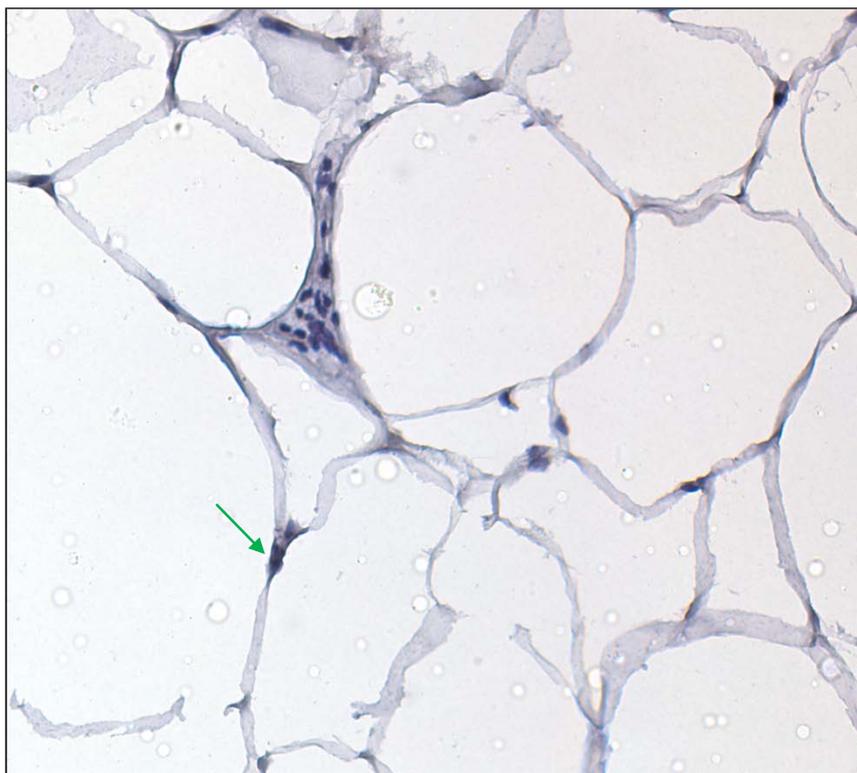
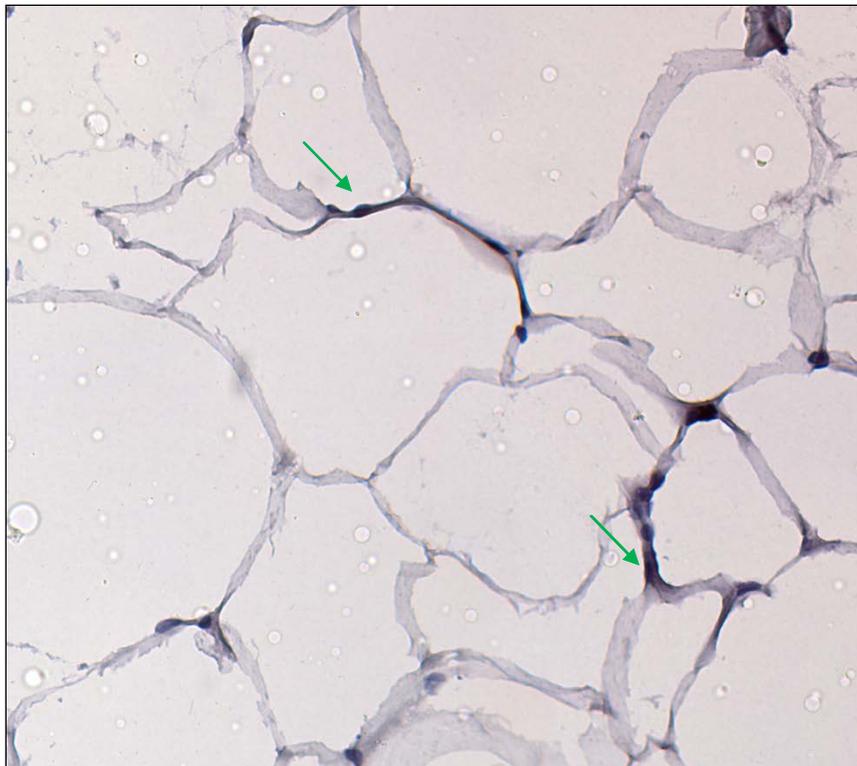


Figure 11. Representation of histological macrophage staining in epididymal adipose tissue. Macrophages are stained in dark black, indicated by arrows.

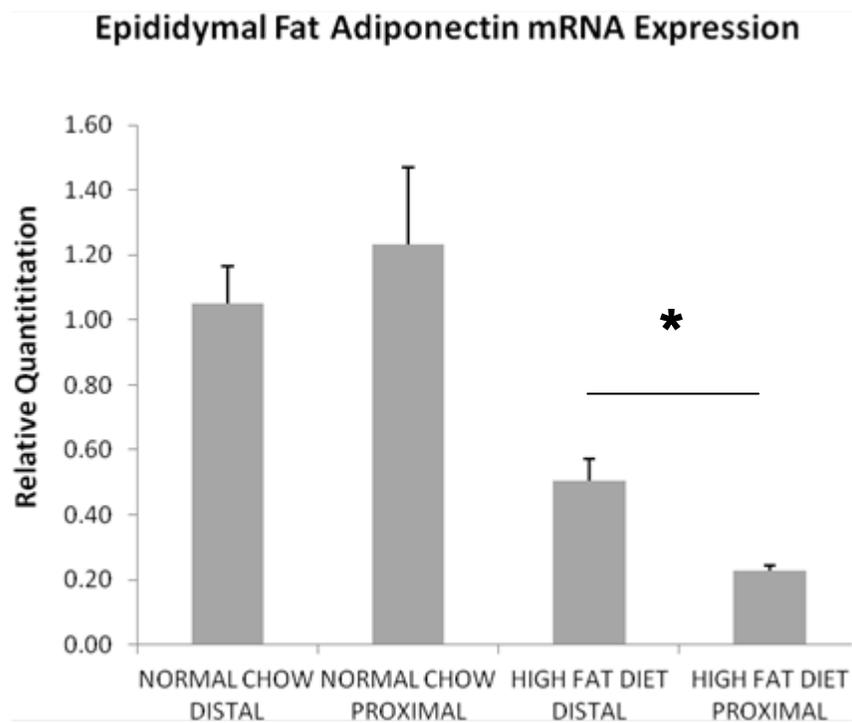


Figure 12. Epididymal adipose tissue adiponectin gene expression in high-fat fed mice and normal-chow fed mice. Comparison of distal and proximal segments of fat pad.

* p - value < 0.001

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