Abstract

Purpose. Peripheral edema is a condition characterized by the accumulation of excess interstitial fluid in distal tissues and commonly manifests in the arms or legs. Untreated complex peripheral edema can progress into chronic lymphedema as impaired fluid drainage and chronic inflammation cause irreversible damage to the surrounding tissue and local lymphatics. The overall goal of our research is to study the effectiveness of osteopathic manipulative medicine techniques (OMT) for the treatment of edema, infection and inflammation. The aim of this study was to evaluate oxazolone (OXA) as a model to induce acute inflammation and lower limb edema in the rat. Specifically, we hypothesized that a single application of OXA would induce a local inflammatory response and induce edema in the lower limb. **Methods.** Female Sprague Dawley rats, weighing 200-250 g, were used for this study. On day 0 the right lower limb was shaved and 750 µl of 5% OXA-acetone solution or 750 µl of a 5% phosphate buffered saline (PBS)-acetone solution vehicle (VEH) was applied to the exposed skin. Lower limb measurements were made at days 0 and 6 using a Vernier caliper. At day 6, the rats were euthanized, and the bilateral hind paws were removed above the calcaneus and weighed. The spleen and bilateral inguinal lymph nodes were removed, homogenized, centrifuged and cells were stained with phycoerythrin (PE) mouse anti-rat granulocytes, fluorescein (FITC) anti-rat CD3, and allophycocyanin (APC) anti-rat CD161 antibodies. The percentage of granulocytes, T cells and dendritic cells were measured by flow cytometry. Data were analyzed by analysis of variance (ANOVA) followed by Tukey-Kramer post-test or by Student's t-test. Comparisons were made between OXA and VEH groups at day 6 post-induction. **Results.** OXA did not induce significant ($p>0.05$) changes in either hind paw thickness or hind paw volume. OXA significantly ($P<0.05$) increased the concentration of macrophages, neutrophils, and dendritic cells, and T cells within inguinal lymph nodes. **Conclusions.** OXA induced an acute local inflammatory response in the draining inguinal lymph nodes. However, as used in this approach, OXA did not induce peripheral edema. In future studies we will investigate alternate strategies to induce lower limb edema in the rat.

OXAZOLONE AS A MODEL TO INDUCE EDEMA IN THE LOWER LIMB OF RATS

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INTERNSHIP PRACTICUM REPORT

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Chapter 1: Background and Literature

The Lymphatic System

The lymphatic system performs multiple functions including tissue fluid homeostasis, lipid absorption, and promoting immune surveillance (Swartz 2001). The lymphatic system maintains tissue fluid homeostasis through a network of morphologically-unique vasculature which act as a one-way transport highway for interstitial fluid to be returned to systemic blood circulation (Swartz 2001). In contrast to the cardiovascular system, the lymphatic system lacks a central pumping mechanism and therefore remains under relatively low pressure. Intrinsic vascular smooth muscle cells in conjunction with extrinsic changes in pressure gradients throughout the body enable the lymphatic system to propel interstitial fluid, called lymph once it enters the lymphatic vasculature, along its length and ultimately into the right atrium of the heart.

Interstitial fluid, which contains plasma proteins, immune cells, cellular debris, inorganic and foreign particulate matter, enters the initial lymphatics through lymphatic capillaries located in interstitial tissue (Swartz 2008). Lymphatic capillaries, composed of a single-cell layer of endothelium, appear collapsed at rest and are extremely permeable because they lack basal lamina. Anchoring filaments extending from the endothelial cells attach the lymphatic capillaries to the extracellular matrix (ECM) of the surrounding interstitial tissue (Swartz 2001). The cooperative integration of the lymphatic capillaries and the ECM facilitates the luminal expansion of the capillary under ECM strain, which creates negative pressure inside of the lumen of the capillary relative to the ECM (Swartz 2001). Interstitial fluid flows down the transient pressure gradients into the lymphatic capillary, and naturally-oscillatory strain on the ECM caused by bodily movement effectively creates a local pumping mechanism at the lymphatic capillary-ECM interface.

Lymphatic capillaries give way to segmented lymphatic vessels lined with smooth muscle cells, and lymph flows from capillaries into the larger lymph vessels. The segments of the lymphatic vessels are functional units called lymphangions. Lymphangions are separated by semilunar valves that prevent the backflow of lymph, thus ensuring one-way transport through the low-pressure system of the lymphatics (Swartz 2001). Lymphangions act together as a series of pressure chambers (Gashev 2002). Each lymphangion accumulates lymph until enough pressure builds to force open the valve to the next lymphangion. The smooth muscle cells lining the lymphatic vessels also respond to the stretch forces of the accumulating lymph, and initiate contractions to pump lymph into the downstream lymphangion. Lymphatic smooth muscle cells can respond to circulating or released humoral factors (Muthuchamy 2008). Extrinsic forces such as stretch, compression, musculoskeletal movement, respiration, and changes in blood pressure, can also modulate lymph flow (Gashev 2002). Malformation, blockage, or damage to the lymphatic vessels, which disrupts the ability of the lymphatic vasculature to effectively pump, can lead to diseases such as edema or lymphedema.

Peripheral Edema

Interstitial fluid naturally forms as part of the microvascular exchange across capillaries and venules that allows for the delivery of nutrients, hormones, other cell products to, and waste products from, the tissues. The rate of interstitial fluid formation depends on properties of the capillary walls, hydrostatic pressure, and protein concentrations in the blood and interstitium, and is conventionally described by the Starling Equation

$$J_v = L_p S[(P_c - P_{if}) - \sigma(COP_c - COP_{if}]$$

where J_v is the volume filtration rate (cm^3/s), L_p is the hydraulic permeability of the capillaries ($cm \cdot mmHg^1 \cdot s^1$); S is the surface area available for filtration (cm^2); σ is the capillary reflection coefficient (dimensionless, with values between 0 and 1); P_c and P_{if} are the hydrostatic pressures (mmHg) in the blood capillary and interstitial compartments, respectively; and COP_c and COP_{if} are the colloid osmotic pressures (mmHg) in the capillary and interstitial compartments, respectively (Wiig 2012). Interstitial buffering mechanisms, including these colloid and osmotic forces, structural changes in the microvascular endothelium and interstitium, and lymph flow, interact to maintain normal interstitial fluid volume (~20% of body weight) (Wiig 2012). However, increased capillary permeability or capillary pressure, or decreased plasma oncotic pressure, can disrupt tissue fluid homeostasis and lead to the accumulation of excess interstitial fluid – a condition known as edema.

Peripheral edema is the pathophysiological condition characterized by the accumulation of excess interstitial fluid (edema) in the distal tissues perfused by peripheral circulation. Consequently, patients experience peripheral edema in the arms or legs that clinically manifests as painful swelling of the tissues (Cho 2002). Peripheral edema can range from a mild and temporary condition, which spontaneously resolves, to a severe and chronic symptom of an underlying disease (Tabibiazar 2006). The etiology of peripheral edema can be linked to a variety of causes, including, but not limited to, increased water retention, venous insufficiency, cardiopulmonary disease, kidney disease, infection, genetic susceptibility, physical trauma, and pharmaceutical side effects (Cho 2002, Frank 2007). What each of these disease processes share is their ability to disrupt the homeostatic filtration of intravascular fluid into the extravascular interstitial space (Cho 2002). For example, congestive heart failure increases venous pressure,

which leads to increased capillary hydrostatic pressure and increased filtration out of the vascular space (Cho 2002). If left untreated, excess edematous fluid promotes inflammation, adipose deposition, and fibrosis, which progressively damages surrounding tissues (Cucchi 2017). Tissue damage may include the initial and collecting lymphatic vasculature, and sufficient fibrosis may develop into lymphatic insufficiency and the formation of lymphedema. Further research of the relationship between peripheral edema and the lymphatic system will provide insight into the mechanisms of interstitial fluid overload and inflammation leading to lymphatic insufficiency, and possible new modes of intervention for peripheral edema and lymphedema.

Lymphedema

Lymphedema is the pathophysiological result of chronic lymphatic vessel insufficiency, and is a condition distinct from, but similar to, peripheral edema. While peripheral edema is caused by an imbalance in the net filtration of vascular fluid into the extravascular space leading to an excess accumulation of interstitial fluid, lymphedema is characterized by damaged or dysfunctional lymphatic vasculature leading to the excess accumulation of interstitial fluid. Lymphatic insufficiency, the lack of lymphatic pumping on either the local or systemic levels, causes protein-rich interstitial fluid to accumulate in the interstitial tissue space and form edema. The protein-rich interstitial fluid promotes adipose deposition, and chronic fluid stasis causes degradation of interstitial tissue as protein imbalance, apoptotic debris and foreign particles provoke an immune response (Cucchi 2017). This immune response worsens damage to lymphatic vasculature as tissue fibrosis and further adipose deposition occur in response to inflammation (Mortimer 2014). Tissue damage, caused by similar mechanisms in progressive peripheral edema, has been shown to deleteriously affect the lymphatic vasculature and promote the development of lymphedema (Maclellan 2014). Therefore, lymphedema is a progressive

condition that must be treated early in its pathophysiological process to prevent further progression to increasing severe stages of lymphedema.

Lymphedema typically manifests as swelling in the affected parts of the body. Similar to peripheral edema, lymphedema is especially evident in the peripheral limbs or areas of the body upstream of the affected lymphatics (Ridner 2013). Primary lymphedema is rare and caused by congenital abnormalities in the formation of lymphatic vasculature. Approximately 1 in 6000 people will develop primary lymphedema (Heinig 2017). In contrast, secondary lymphedema is the result of injury-related damage to lymphatic vasculature, and is, in the developed world, primarily a complication of surgery requiring lymph node or vessel transection with a reported incidence ranging from 12-78% (*Lymphoedema Framework* 2006). Furthermore, lymphedema may manifest as a comorbidity to infection, cardiopulmonary disease, and obesity (Rockson 2010), while studies have shown evidence for an additive effect between obesity and lymphedema in the pathophysiological progression of lymphatic vascular damage and insufficiency (Cucchi 2017). Currently, a cure for lymphedema eludes discovery due to a lack of research into the mechanism of the lymphatic system and its dysfunction relative to other organ systems, most notably its closest analog – the cardiovascular system (Mortimer 2014).

Psychosocial morbidities and pain coincide with the physical symptoms of lymphedema. Increased limb size can reduce mobility, and chronic edema requires constant and lifelong care (Leung 2015). Cost of healthcare and quality of life are serious considerations that must be addressed when planning appropriate intervention. Current standards of care recommend exercise and weight loss in combination with complex decongestive therapy (CDT) to alleviate symptoms of lymphedema and prevent its progression to more severe stages of disease (*Lymphoedema Framework* 2006). Exercise, CDT and weight loss have been shown to be

effective in reducing limb size and the severity of lymphedema which has not progressed too far. However, exercise and weight loss sufficient to prevent the progression of lymphedema may not be feasible for certain patients, especially those where serious comorbidities are present, such as morbidly obese patients (Deura 2015). CDT also possesses limitations, such as lapses in compliance or severity of damage to skin and tissue directly associated with insufficient lymphatic vasculature (Deura 2015). Osteopathic manipulative techniques (OMT), specifically lymphatic pump techniques (LPT) aimed at enhancing lymphatic function, may provide an alternative physical therapy with the ability to overcome limitations inherent to conventional CDT (Chikly 2005). The development of new animal models that establish the formation of feasible and clinically-relevant levels of peripheral edema with lymphatic insufficiency may increase understanding and support for existing and new therapies targeting lymphedema.

The Role of the Lymphatic System during Inflammation

The transport of lymph allows the lymphatic system to provide immune surveillance and trafficking of immune cells throughout the body. Interstitial fluid movement from the tissue into the lymphatic system as lymph continuously provides information about the tissue to the immune system. The lymph vessels transport antigens, toxins, cytokines, chemokines, apoptotic cells and antigen-activated dendritic cells (DC) from tissues to the lymph nodes (Swartz 2007). Within the lymph nodes, antigen-activated dendritic cells present tissue antigens to T cells and promote the activation and differentiation of T cells into effector cells. Once in the bloodstream, these activated lymphocytes return to the tissue where they perform their effector functions (Swartz 2001). The delivery of antigens and DCs to the draining lymph nodes is essential to promote

adaptive immunity; therefore, blockage or disruption of the lymphatic vessels may result in altered immune responses.

During inflammation, the lymphatic vessels are believed to reduce edema and promote the migration of immune cells to the downstream lymph nodes (Lachance 2013). In response to inflammation, immune cells and lymphatic endothelial cells release growth factors, cytokines and chemokines that induce lymphatic remodeling (Aebischer 2014, Lachance 2013). The process of lymphatic endothelial cell (LEC) proliferation and regression promotes vessel dilation and leakiness which lowers the ejection of lymph fluid before newly organized lymphatic vasculature resumes normalized lymph transport (Huggenberger 2011). The transient reduction in lymph flow also impedes the trafficking of dendritic cells through the lymphatic vessels, which impairs T cell priming within the draining lymph nodes (Liao 2012). Thus, a healthy lymphatic system is vital to promote an immune response during infectious and inflammatory disease.

In the presence of diseases that promote inflammation and affect lymphatic function, the ability of lymphatic remodeling to effectively mediate an immune response and promote edematous drainage remains poorly understood. While lymphangiogenesis provides a compensatory mechanism for fluid overload, excess lymphangiogenesis mediated by CD4+ T cells and macrophages have been found to drive the pathogenesis of lymphedema (Ogata 2016). Furthermore, CD4+ T cells have been shown to mediate fibrosis in response to lymphatic fluid stasis (Zampell 2012, Gousopoulos 2016). However, the exact component(s) present in the lymph fluid that initiates the pathophysiological progression towards lymphedema is unknown (Cucchi 2017). Therefore, models of inflammation that induce edema and lymphatic

insufficiency may provide deeper insights into the processes mediating the formation of lymphedema.

Oxazolone-induced Contact Hypersensitivity model

Oxazolone (OXA) is a chemical compound known to be a potent irritant capable of inducing irritant contact dermatitis after a single exposure and allergic contact dermatitis after repeated exposures (Man 2008). OXA has been used extensively in animal models of allergic contact dermatitis to study the cytokine release patterns, innate and adaptive immune responses, and efficacy of pharmaceutical therapies for inflammatory skin disease (Igney 2006, Zollner 2005). OXA induces inflammation and a Type IV hypersensitivity response through its function as a hapten. Haptens are small, non-biologic molecules that are incapable of inducing a specific immune response on their own but become immunogenic after covalently binding with a carrier protein (Chipinda 2011). Induction of a contact hypersensitivity occurs after initial exposure to a hapten chemical, during which haptenated-proteins sensitize the immune system by stimulating antigen-presenting cells, such as dendritic cells. In this sensitization phase, antigen-presenting cells take up and process the haptenated-protein, migrate to secondary lymphoid tissue, and present hapten-antigen via major histocompatibility complex I/II to naïve T cells (Erkes 2014). Presentation of hapten-antigen to naïve CD4+ T helper cells primes the adaptive immune system and activates T cell maturation and differentiation into hapten-specific effector and memory T cells. During the elicitation phase, subsequent contact with the sensitizing-hapten elicits hapten-specific memory T cell proliferation and effector T cell migration to the site of elicitation, where the cells promote robust inflammatory response that results in damage to the surrounding tissue (Erkes 2014).

Topical application of OXA in solution promotes an acute, localized type 1 cytokine response. OXA-induced chronic delayed-type hypersensitivity models result in a primarily Type 2 T helper cell-mediated dermatitis and measurable edema limited to the area upon which the agent was topically applied (Webb 1998). Therefore, OXA-induced models of allergic contact dermatitis may be used to elucidate the macrophage and CD4+ T cell-mediated processes of fibrosis leading to the development of lymphatic vascular insufficiency. Upon contact, OXA penetrates the skin and interacts with keratinocytes by binding with their surface proteins. Haptenation of keratinocytes causes the cells to release the pro-inflammatory cytokines IL-1β, IL-18, TNF-α, and GM-CSF, which activate Langerhans cells and dermal dendritic cells to migrate begin the process of priming a cell-mediated adaptive immune response (Erkes 2014). Haptenation of the keratinocytes also promotes the release of "danger signals," such as hyaluronic acid, extracellular matrix ligands for Toll-like receptors, prostaglandin E2, reactive oxygen species, heparin sulfate, B defensins, and fibrinogen, which function in innate immune activation (Erkes 2014).

OXA-induced contact hypersensitivity models have provided deeper insight into the pathophysiology of allergic contact dermatitis (Zollner 2005). OXA has also been utilized in both chronic and acute models of induced contact dermatitis to investigate the effects of localized inflammation and subsequent edematous fluid formation on lymphatic function. The results of the OXA-induced irritant contact dermatitis murine model described by Liao et al., and the OXA-induced delayed-type hypersensitivity (DTH) rodent model used by Mendez et al., indicate that OXA-induced inflammation and the peripheral edema formed as a result can cause or worsen lymphatic drainage.

Liao et al. describe a murine model of irritant contact dermatitis that induced lymphatic impairment using a single application of OXA without subsequent OXA-challenge. Naïve mice received a single application of 50 microliters of 4% OXA solution in acetone on shaved skin at the areas of the inguinal and brachial lymph nodes on one side of the abdomen (Liao 2006). Interstitial fluid accumulation and lymphatic function were then quantified 4 and 7 days after OXA challenge by Evans blue dye (EBD) tracer injected into the lower limb (Liao 2006). EBD accumulation in the tissue surrounding the peripheral lymph nodes was evaluated 6 hours later by optical imaging of EBD fluorescence under ultraviolet light (Liao 2006). EBD dye accumulation in the peripheral lymph nodes was less in day 4 mice versus control mice, and the tissue surrounding the lymph nodes appeared edematous, while EBD accumulation and the associated edema was nearly recovered in day 7 mice (Liao 2006). Liao et al. also quantified dendritic cell access into the peripheral lymph nodes using fluorescein (FITC) skin painting and flow cytometry to tag $CD11c^+$ dendritic cells. OXA-immunized naïve mice at day 0, day 1, day 2, et cetera continuing to day 14 received FITC-skin painting on the shaved skin of their flanks. 24 hours after skin painting, peripheral lymph nodes were collected, and dendritic cell concentration quantified by flow cytometry (Liao 2006). Newly activated dendritic cell concentration in the peripheral lymph nodes was reduced transiently reduced from days 1-5 after OXA, before recovery and enhanced concentration of dendritic cells compared to non-immunized mice out to day 14 (Liao 2006). The results of their study indicate that a single application of OXA induced localized edema and lymphatic insufficiency in naïve mice, presumably through the innate immune response, since their model did not utilize sensitization and subsequent challenge steps to elicit an adaptive immune response seen in conventional models of OXA-induced contact dermatitis.

Mendez et al. describe a conventional DTH-model utilizing chronic application of topical OXA to rat forelegs after sensitization to OXA to induce peripheral edema. The rats were sensitized by abdominal painting seven days prior to OXA challenge, and the rats received three applications of OXA to their forelegs over a time course of nine days for a total of four applications of OXA (Mendez 2012). Lymphatic function and interstitial fluid transport were quantified 12 days after initial application of OXA by a fluorescent tracer injected into the forelimbs, and physiological measurements were taken using digital image and software analysis of the rat forelegs (Mendez 2012). Mendez et al. found that OXA induced significant edema compared to untreated rats.

Together, the results of both studies in rats and mice, indicate that OXA-induced inflammation and the peripheral edema formed as a result can cause or worsen lymphatic drainage. It remains unknown whether certain therapies that are proposed to improve lymphatic function, such as OMT, can improve lymphatic function following OXA-induced lymphatic insufficiency. By taking advantage of previously established LPT adapted to a rodent model (Huff 2010) and combining with OXA-induced model of lymphatic insufficiency described by Liao et al., a potentially feasible, novel animal model might be created to study the efficacy of LPT in the context of inflammation, edema, and lymphatic insufficiency. Furthermore, the irritant contact dermatitis model described by Liao et al. provides a starting point targeting the innate immune response and the processes of inflammation and sensitization which prime a cell-mediated adaptive immune response. Future studies applying modifications to a successful adaptation the Liao et al. OXA-induced contact dermatitis model combined with rodent LPT could include OXA-challenge and chronic exposure to OXA to model the progressive CD4+ T cell-mediated inflammatory processes implicated in the pathogenesis of lymphedema. Therefore,

the development of an OXA-induced contact dermatitis model may be useful to study interventions proposed to improve lymphatic function following an inflammation-mediated lymphatic insufficiency, such as those found in persistent peripheral edema or obesity-related lymphedema.

Chapter 2: Research Project

I. Specific Aims

The purpose of this research project was to investigate the efficacy of a rat model of oxazolone-induced acute skin inflammation to induce the formation of peripheral edema in the rat hind paw. Specifically, we hypothesized that a single application of OXA to the lower limb of rats would 1) induce a local inflammatory response and 2) induce peripheral edema in the hind paw of rats. By the completion of this study, we hope to develop a rat model for use in future studies that will study the effectiveness of manual therapies at reducing inflammation and edema.

II. Significance & Innovation

Peripheral edema is a manageable condition that manifests as a result of interstitial fluid imbalance. Management of peripheral edema includes interventions that enhance lymphatic system function alongside those aimed at treating the underlying cause of edematous formation. However, peripheral edema is a potential complication of serious and chronic disease states, including those placing the greatest burden on our healthcare system today: cardiac disease, obesity, cancer and infection (Mortimer 2014). The difficulties in treating these disease states may lead to persistent peripheral edematous fluid accumulation and chronic inflammation that is resistant to or contraindicated with current therapies. Uncontrolled peripheral edema overloads the lymphatic system, and the prolonged stasis of edematous fluid causing chronic inflammation may progress to lymphedema. Chronic lymphedema places a lifelong burden on patients' quality of life and is associated with chronic pain, potential psychosocial and mobility complications, and increased cost of care. Currently, no cure exists for chronic lymphedema, and treatment options remain palliative in nature. Therefore, new and alternative interventions aimed at

relieving edematous fluid overload and halting the progression of chronic inflammation, adipose deposition, and fibrosis must be developed. Investigations are being made into potentially curative interventions such as lymphatic vessel transplant and promotion of lymphangiogenesis, but there remains a lack of knowledge regarding non-invasive manipulative therapies that propose an alternative to current physical therapies. Our animal model offers a new approach for pre-clinical study of these manipulative therapies, specifically OMT, by utilizing a species of appropriate size for which these manipulative therapies have already been adapted combined with a previously studied model of OXA-induced contact dermatitis promoting edema formation and lymphatic insufficiency. Furthermore, by focusing on the relationship between inflammation, peripheral edema, and lymphatic vessel function, we aim to enhance our understanding of the mechanisms provoking the progression of peripheral edema, while establishing support for new and existing therapies and interventions for edema and lymphedema.

III. Materials and Methods

Animals

Thirty-two adult female Sprague Dawley rats weighing between 200-250 grams were purchased from Envigo RMS (Indianapolis, ID). Rats were divided randomly into 2 groups: experimental OXA (n = 13) and vehicle-control (VEH; n = 19). Rats were housed in a temperature and light controlled barrier room in facilities of the University of North Texas Health Science Center Department of Laboratory Animal Medicine. All rats were housed separately in individual cages with access to food and water ad libitum. All rats were studied

under a protocol approved by the University of North Texas Health Science Center Institution Animal Care and Use Committee.

Preparation of solutions

Two topical solutions were used in this experiment: the experimental solution of 5% (w/v) OXA (Sigma, St. Louis, MO) in acetone and the VEH solution of 5% (v/v) phosphate buffered saline (PBS) in acetone. VEH solution were used to control for the effects of anesthesia, shaving, and topical application of acetone. Topical solutions were prepared the morning of use and stored in ice until use.

Oxazolone-induced Acute Skin Inflammation Model

To test our hypothesis, we modified the mouse oxazolone-induced skin inflammation model described by Liao et al. (Liao 2006). On day zero, rats were anesthetized with 2.5% isoflurane mixed with oxygen gas administered by an induction chamber and transferred to continuous 2.5% isoflurane mixture administered by nose cone for the duration of the surgery. Anesthetized rats were laid supine and their right lower limb close-shaved with a 1.5 mm clipper blade. After shaving, initial measurements of the right hind paw and lower limb were taken.

After measurement, rats received either OXA solution or VEH solution according to their group assignment. For consistency, the same experimenter who measured and shaved the lower limb also applied solutions to the lower limbs. The identities of the rats and their group assignments were blinded to the experimenter handling the rats, and notes were taken by another experimenter. Using a micropipettor, 750 microliters of assigned-solution were applied to the shaved skin of the right lower limb, but not the skin of the hind paw. Rats remained under

anesthesia until the area of the skin where solution was applied had completely dried. Rats were then weighed before being returned to their cages.

Rats underwent daily monitoring for adverse reactions to anesthesia and OXA or VEH solutions. Changes in the skin, such as inflammation, discoloration, and ulceration were also recorded. On day 6, rats were euthanized using a combination of ketamine/xylazine overdose followed by diaphragm ligation and cardiac puncture with blood collection. After euthanasia, final measurements of the right hind paw and lower limb were taken, and the right and left hind paws removed above the calcaneus bone with surgical scissors and weighed. Finally, 5 bilateral inguinal lymph nodes and the spleen were collected and prepared for analysis by immunoassay and flow cytometry.

Paw Thickness and Lower Limb Volumes

Measurements of the thickness of the lower limb were taken at 4 points: across the metatarsal-phalangeal joints of the hind paw, the ankle, the midpoint of the tibia, and the midpoint of the femur. Thickness measurements were taken by Vernier caliper 3 times and averaged at each point of measurement to reduce error caused by deviations in measurement. The distance (h) between the thickness measurements taken at the hind paw and ankle, paw-to-ankle, and the distance (h) between the thickness measurements taken at the ankle and midpoint of the tibia, ankle-to-tibia, were used to calculate the volume of those respective segments of the lower limb. The volume of each segment was calculated using the truncated cone formula, $Volume = \frac{1}{3}\pi(r_1^2 + r_1 r_2 + r_2^2)h$, commonly used to clinically assess lymphedema (Sitzia 1995). On day 6, following euthanasia and the final thickness measurements, the treatment

(right) and untreated contralateral (left) hind paws of all rats were removed above the calcaneus bone and weighed for comparison.

Quantification of the Inflammatory Response

On day 6, rats were euthanized by ketamine/xylazine overdose, followed by diaphragmatic incision and cardiac puncture procedures. Blood was collected during cardiac puncture with a 10-milliliter syringe and dispensed into EDTA-treated vacutainers prior to being placed on a mechanical rocker to further prevent coagulation. Spleen and 5 each of the draining and contralateral lymph nodes were collected and placed into 5 milliliters and 3 milliliters of wash media, respectively.

Blood samples were then centrifuged at 1200 G, 4ºC, for 15 minutes, and the plasma component collected into Eppendorf tubes by micropipettor. Spleen and lymph node samples were homogenized using mesh boats and mashers under a sterile hood. Spleen and lymph node homogenates were then centrifuged at 800 G, 4ºC, for 10 minutes. After centrifugation, supernatant was collected in Eppendorf tubes and placed with blood plasma samples into storage at -20°C for future immunoassay analysis with rat TNF ELISA (BD Biosciences, San Diego, CA). The remaining splenic and lymph node cells were then washed with 10 and 50 milliliters of wash media, respectively, and centrifuged, followed by another cycle of wash and centrifugation. Cells were then suspended in wash media, and the total number of leukocytes were enumerated by trypan blue exclusion.

Cells were then diluted in staining buffer (2% fetal bovine serum (Hyclone Labs, Logan, UT) in PBS) to bring samples to $1x10^7$ cells per milliliter. 100 microliters of each sample were pipetted in duplicate into a 96-well plate and centrifuged at 1500 rpm, 4ºC, for 5 minutes.

Supernatant was then removed, and the remaining cell pellet broken up and stained with 100 microliters of fluorophore-antibody dye by micropipettor. Samples were stained with specific dyes to differentiate between dendritic cell, macrophage, neutrophil, and T cell subpopulations. allophycocyanin (APC) anti-rat CD161 was used to target dendritic cells; phycoerythrin (PE) mouse anti-rat granulocyte was used to target macrophages and neutrophils; fluorescein (FITC) anti-rat CD3 was used to target T cells. After application of dye, samples were placed in the dark for 30 minutes, and then centrifuged. Supernatant was then discarded, and samples were washed with 200 microliters of staining buffer by micropipettor. Samples were washed once more, and then fixed with 200 microliters of 0.5% paraformaldehyde in PBS. Samples were pipetted from their plate into flow tubes containing 300 microliters 0.5% paraformaldehyde in PBS, which were then covered in aluminum foil and stored at 4ºC. The percentage of dendritic cells, macrophages and lymphocytes in the spleen and inguinal lymph nodes were measured by flow cytometry 2 days after fixation.

Statistical Analysis

Statistical analysis of the lower limb measurements, including paw-to-ankle and ankle-to-paw volumes, was performed using one-way analysis of variance (ANOVA) to compare differences between the OXA and VEH groups and the inter-group differences between their baseline measurements taken at day 0 to their final measurements at day 6. The Tukey-Kramer multiple comparisons post-hoc test was used to determine which groups and at which time points experienced significant differences. Statistical analysis of the hind paw weights was performed using one-way ANOVA to compare the differences between OXA and VEH groups and their contralateral hind paws, followed by the Tukey-Kramer post-hoc test, which was used to

determine which groups experienced significant differences. Statistical analysis of the lymph node flow cytometry data was also performed using one-way ANOVA, followed by the Tukey-Kramer post-hoc test to compare differences in leukocyte subpopulations between draining and contralateral inguinal lymph nodes. Statistical analysis of the percent change in limb lower limb thickness from day 0 to day 6 was performed by Student's t-test. Student's t-test was used to compare the differences in leukocyte populations between OXA and VEH groups in the spleen. In all comparisons, $P \leq 0.05$ was considered significant. Data were analyzed and graphs produced using GraphPad Prism software (La Jolla, CA).

IV. Results

Clinical signs

Within 24 hours of topical application with the OXA solution, the OXA rats developed right lower limb inflammation characterized by reddish-yellow discoloration and mottling of the skin compared to VEH rats. No apparent pain or change in behavior and activity accompanied the OXA phenotype, and OXA rats continued to favor the use of both hind paws equally. Skin inflammation was present in the OXA group until day 6 of the study.

Peripheral Edema

Thickness of the lower limb measured across each of the 4 locations was not significantly ($P > 0.05$) different between the VEH and OXA treatment groups (see Figures 1A, 2A, 3A, and 4A). The percent change in lower limb thickness measured across each of the 4 locations was calculated for each individual rat to account for morphological differences between individual rats when comparing lower limb thickness between the VEH and OXA treatment groups. For

each individual rat, the change in lower limb thickness measured across each of the 4 locations was calculated as the difference between the thickness-value measured on day 0 to day 6 by subtracting the baseline thickness-value measured on day 0 from the final thickness-value measured on day 6. The percent change for each individual rat was then calculated by dividing the difference between the thickness-value from day 0 to day 6 by the baseline thickness-value on day 0, and then multiplying by 100. The percent change of lower limb thickness across each of the 4 locations were then analyzed and compared between the VEH and OXA treatment groups. The percent change in ankle thickness from day 0 to day 6 was significantly ($p < 0.0214$) increased in the OXA group compared to the VEH group (see Figure 2B). The percent change in paw thickness, tibia thickness, and femur thickness from day 0 to day 6 was not significantly different between the VEH and OXA treatment groups (see Figures 1A, 3A, and 4A). Paw weights between the VEH and OXA groups and their contralateral hind paws were not significantly different (see Figure 5). Lower limb volume and percent change of the paw-to-ankle and ankle-to-tibia calculated by the truncated cone formula was not significantly different between the VEH and OXA treatment groups (see Figures 6 and 7).

Flow Cytometry

In the draining lymph nodes, the concentration of DC, macrophage, neutrophil, and T cell populations were significantly different in the OXA group (see Figure 8A-D). DC, macrophage, neutrophil, and T cell populations in the draining lymph nodes of the OXA group were significantly ($p < 0.0001$, $p < 0.0001$, $p = 0.0002$, $p= 0.0002$, respectively) increased compared to the DC, macrophage, neutrophil, T cell populations in the VEH group draining lymph nodes and both the OXA and VEH group contralateral lymph nodes (see Figure 8A-D). Multiple

comparisons analysis revealed no significant ($P > 0.05$) difference in leukocyte populations between VEH group draining lymph nodes and VEH group contralateral lymph nodes, VEH group draining lymph nodes and OXA group contralateral lymph nodes, or VEH group contralateral and OXA group contralateral lymph nodes. As a percentage of the total leukocyte population in the draining lymph nodes, the subpopulation of DCs was significantly increased ($p = 0.0041$) in the OXA group compared to the VEH group (see Table 1). As a percentage of the total leukocyte population in the draining lymph nodes, the macrophage, neutrophil, and T cell subpopulations were not significantly different between the OXA group and the VEH group (see Table 1).

In the spleen, the concentration of the dendritic cell (DC) populations in the OXA group were significantly decreased ($p = 0.0451$) compared to the VEH group (see Figure 9A). Macrophage, neutrophil, and T cell populations in the spleen were not significantly different between OXA and VEH groups, though the populations were decreased in the OXA group compared to the VEH group (see Figure 9B-9D). The percentage of the DC subpopulation out of the total number of leukocytes in the spleen was significantly decreased ($p < 0.009$) in the OXA group compared to the VEH group (see Table 2). The was a significant increase ($p = 0.0012$) in the neutrophil subpopulation as a percentage of the total number of leukocytes in the spleen in the OXA group compared to the VEH group (see Table 2). There was not a significant difference in the percentage of the macrophage and T cell subpopulations out of the total number of leukocytes in the spleen between the OXA and VEH groups (see Table 2).

V. Discussion

The goal of this project was to adapt a specific contact dermatitis mouse model of OXA-induced edema and lymphatic insufficiency for use with rats. The treatment group received OXA solution and the control group received VEH solution, while the left lower limb of either group acted as the healthy intra-individual control. OXA was expected to induce peripheral edema formation in the hind paw and decrease the lymphatic function of the lower limb to which it was applied, with the null hypothesis stating that OXA does not induce peripheral edema in the hind paw or change the lymphatic function of the lower limb to which it was applied.

The change in physical appearance in the OXA group compared to the VEH group on day 1 indicates that OXA had a moderate inflammatory effect on the skin that was separate from the effects that might have been caused by anesthesia, shaving or the application of acetone. However, the skin discoloration was not associated with discomfort, debilitation, or abnormal behavior, nor was it determined to be erythema, which is an indication of inflammation and potentially of peripheral edema. The lack of significant discomfort and debilitation may have contributed to preventing the formation of peripheral edema because skeletal muscle movement is an extrinsic force known to enhance lymphatic function. These results are consistent with a single application of OXA acting through an innate immune response in naïve animals and represent a limitation of a model of OXA-induced irritant contact dermatitis to induce the robust inflammatory response associated with adaptive immunity.

The lack of significant differences in the hind paw thickness of the OXA group compared to the VEH group indicate that OXA did not induce significant peripheral edema in the hind paw of the OXA group. Furthermore, there was not a significant difference in the paw weights of the OXA and VEH groups, nor was there a significant difference between the treatment (right) paw

weight and the contralateral paw weight, which acted as the healthy intra-individual control, within the OXA and VEH groups. Peripheral edema begins in the distal extremity, in rats the hind paw of the lower limb, and the lack of significant difference between the OXA treatment paw and its contralateral intra-individual control, as well as the VEH treatment paw, further indicate the lack of edematous fluid formation. There was not a significant difference in the ankle thickness of the OXA group compared to the VEH group, indicating OXA did not induce peripheral edema in the ankle. However, there was a significant difference in the percent change of ankle thickness in the OXA group compared to the VEH group, which may indicate a morphological change in the tissue surrounding the ankle. Future directions in a modified version of this model could include histologic analysis of the lower limb tissue to determine specific morphological changes as a result of OXA, especially if edema is present. There were no significant changes in the thickness of the tibia and femur between the OXA and VEH groups. OXA did not induce significant changes in the volume of the lower limb at either of the two segments calculated by the truncated cone formula, indicating that OXA did not induce significant peripheral edema in the lower limb. While edema formation was predicted to form in the hind paw, the tibia and femur thicknesses and lower limb volumes indicate that a single dose of topical OXA was insufficient to promote edema formation in the localized area of OXA application.

DC, macrophage, neutrophil and T cell populations were significantly increased in the draining inguinal lymph nodes of the OXA group compared to the VEH group. Increased leukocytes in the draining lymph nodes of the lower limb to which OXA was applied indicate an inflammatory response associated with OXA application. The significant increase in DC, macrophage and neutrophil populations between the draining and contralateral lymph nodes

within the OXA group provide support for leukocyte migration specifically from the OXA-affected lower limb to the draining inguinal lymph node. The significant increase in the T cell population in the draining lymph nodes may indicate activated-T cell proliferation, which is expected during the sensitization phase of OXA-induced contact dermatitis in response to the antigen-presenting cells migrating from the OXA-affected lower limb and presenting OXA haptens to naïve T cells in the lymph node. Furthermore, the significantly decreased DC population in the spleen may indicate increased circulation of antigen-presenting cells migrating from the spleen to the site of inflammation by following chemokines released by innate immune cells activated by OXA haptens. However, in the VEH group there was a large variation in the concentration of DCs in the spleen, which may indicate the difference measured in the OXA group is significant, but small. The significantly increased percentage of the neutrophil subpopulation in the spleen may indicate an increased mobilization of circulating neutrophils in response to OXA application. The lack of a significant increase or decrease in the leukocyte populations within the draining and contralateral lymph nodes of the VEH group shows that anesthesia, shaving, or acetone application did not contribute to an inflammatory response, and further support OXA as inducing inflammation in the lower limb.

The physical measurements and the inability of a single application of OXA to induce peripheral edema reveal a significant methodological consideration in the decision to challenge naïve rats with a single dose of OXA without a sensitization step. While the rationale for our model was based on the model described by Liao et al., their methodology was unconventional and similar uses of OXA to induce edema and lymphatic insufficiency have not been described in a current review of the literature. Conventional models of OXA-induced contact dermatitis, such as that described by Mendez et al., include a sensitization phase followed by an elicitation

phase to promote a robust cell-mediated adaptive immune response that reliably promotes edematous formation. Our hope was to successfully induce peripheral edema using the Liao et al. model in rats as a starting point from which to study the efficacy of LPT in the context of the initial inflammatory insult and its resultant edema formation and lymphatic insufficiency before moving on to the adaptive immune response. However, future directions will have to consider the limitations of the OXA-induced irritant contact dermatitis model and make potential modifications to the model including a sensitization step and chronic OXA exposure.

VI. Summary and Conclusions

In this research project, we examined the feasibility of a rat model of OXA-induced acute skin inflammation to induce inflammation and the formation of peripheral edema. We demonstrated that topical application of OXA induced a quantifiable inflammatory response. This response is consistent with a study using OXA skin painting in mice (Liao 2006). The formation of inflammation and migration of leukocytes may provide a useful model to study anti-inflammatory therapies. However, as applied in this study, OXA did not induce edema. In future studies, multiple applications of OXA could be used to induce a delayed type hypersensitivity reaction which may result in significant edema formation.

As the lead investigator on this project, I learned how a scientist must be fluent and current in their knowledge of past and ongoing scientific research, how much planning and preparation is needed to conduct an experiment, and how results, whether unexpected or disappointed, and analysis take patience and time. I came into this project without previous experience in scientific research. Over the course of the project, I have learned new skills and techniques such as how to handle live animals, perform tissue necropsy, and use specialized

equipment. I also learned basic laboratory skills such as using a micropipettor, generating a standard curve, and completing ELISAs. I have learned how to search, read and reference scientific literature. I also learned how to present my knowledge and communicate my ideas in writing and public speaking with my colleagues. Most importantly, I have learned that research is the product of many different people working together between their own individual and busy schedules, and that developing the skills and knowledge for scientific research is just as much for completing your own work as it is for convincing your colleagues to collaborate and invest their time in you as well. I have learned that each project has its own story, and through collaboration and dedicated work, my duty as a researcher is to get those stories told.

VII. References

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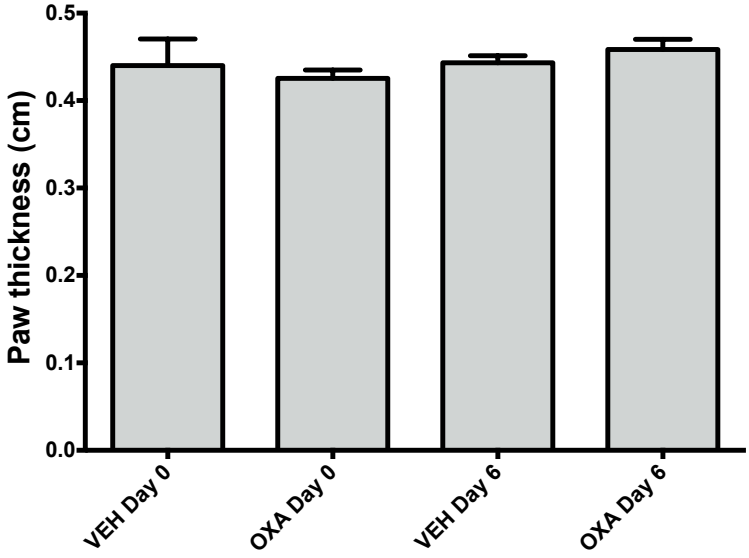

Figure 1A

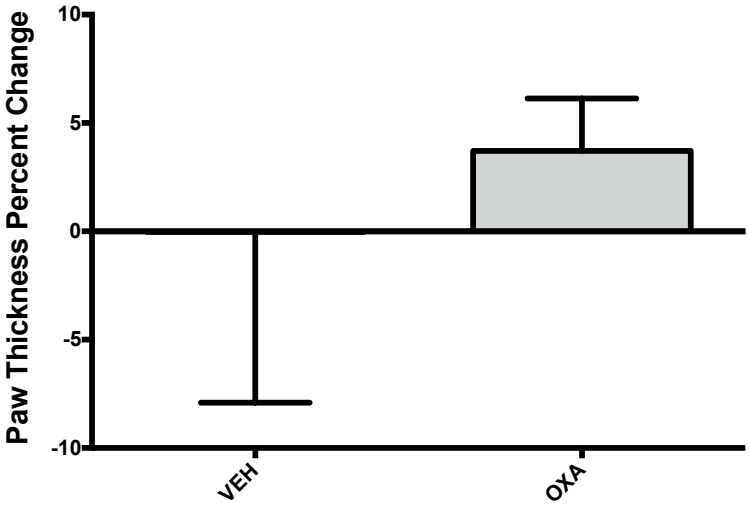

Figure 1B

Figure 1. Paw Measurements. Paw thickness was measured on days 0 and 6 using Vernier calipers. Vehicle (VEH) rats (n=19) received 5% PBS in acetone. Oxazolone (OXA) rats (n=13) received 5% OXA in acetone. Data are means ± SD. A) Data were analyzed by ANOVA followed by Tukey-Kramer post hoc test. B) Data were analyzed by Student's t-test.

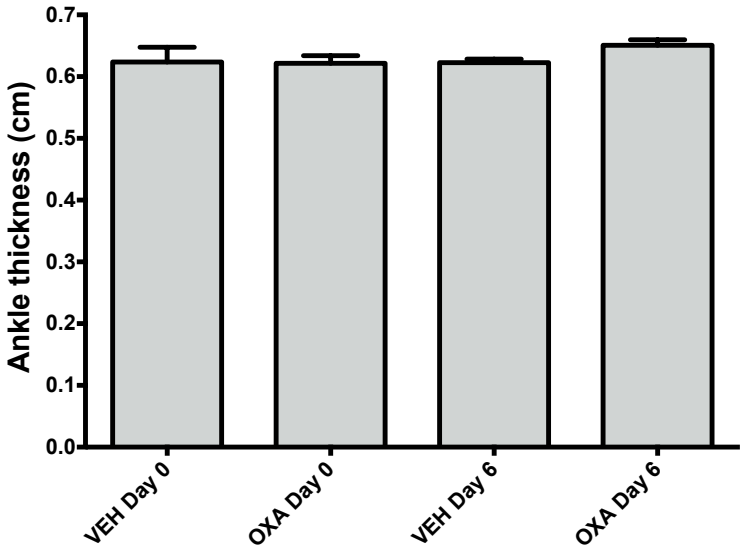

Figure 2A

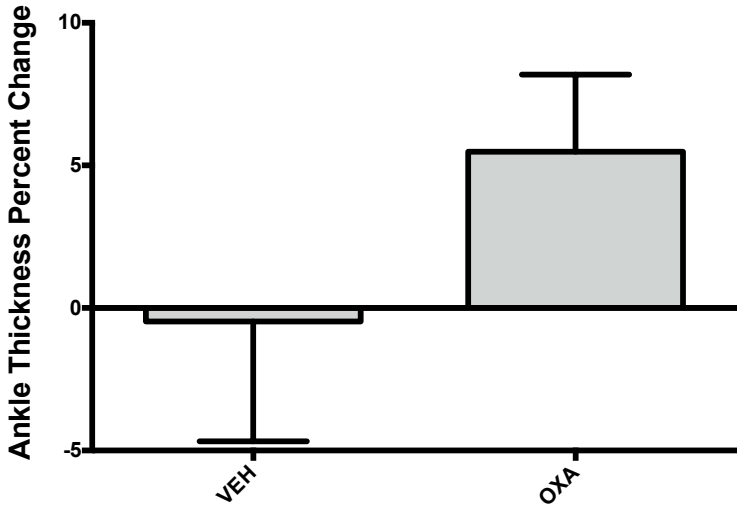

Figure 2B

Figure 2. Ankle Measurements. Ankle thickness was measured on days 0 and 6 using Vernier calipers. Vehicle (VEH) rats (n=19) received 5% PBS in acetone. Oxazolone (OXA) rats (n=13) received 5% OXA in acetone. Data are means ± SD. A) Data were analyzed by ANOVA followed by Tukey-Kramer post hoc test. B) Data were analyzed by Student's t-test. *Significantly ($p<0.05$) increased compared to VEH.

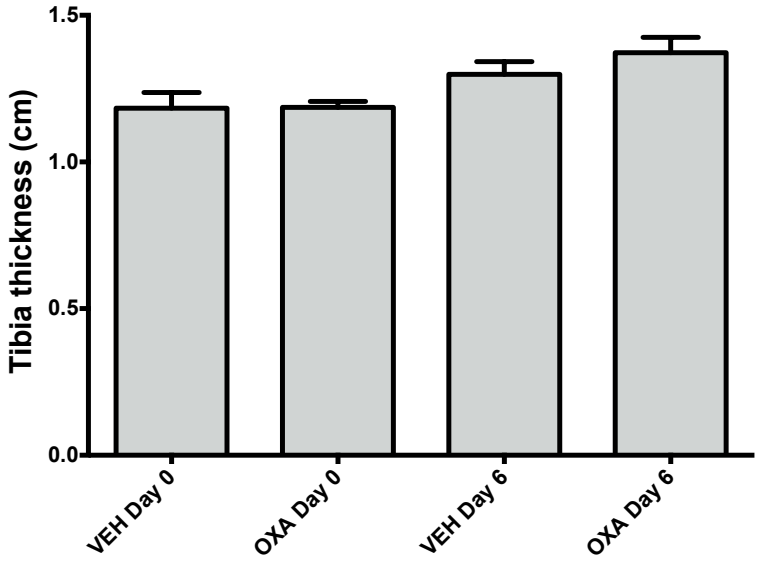

Figure 3A

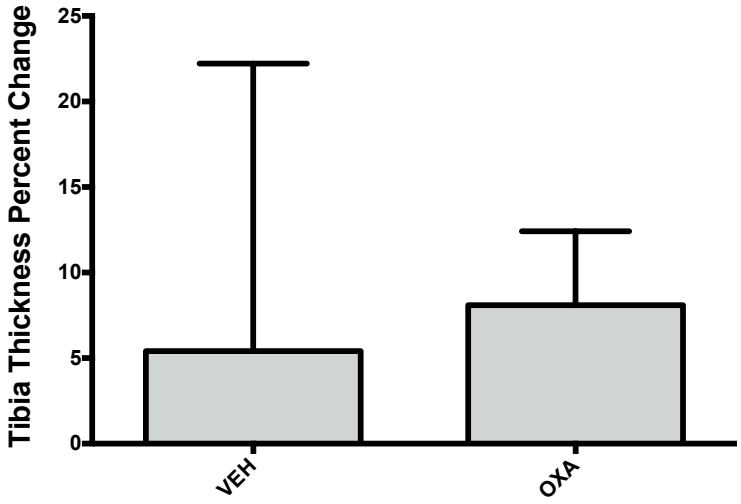

Figure 3B

Figure 3. Tibia Measurements. Tibia thickness was measured on days 0 and 6 using Vernier calipers. Vehicle (VEH) rats (n=19) received 5% PBS in acetone. Oxazolone (OXA) rats (n=13) received 5% OXA in acetone. Data are means ± SD. A) Data were analyzed by ANOVA followed by Tukey-Kramer post hoc test. B) Data were analyzed by Student's t-test.

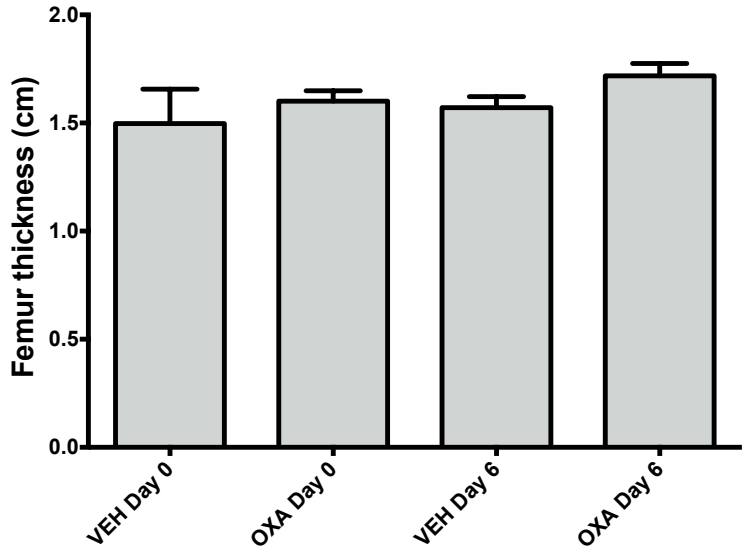

Figure 4A

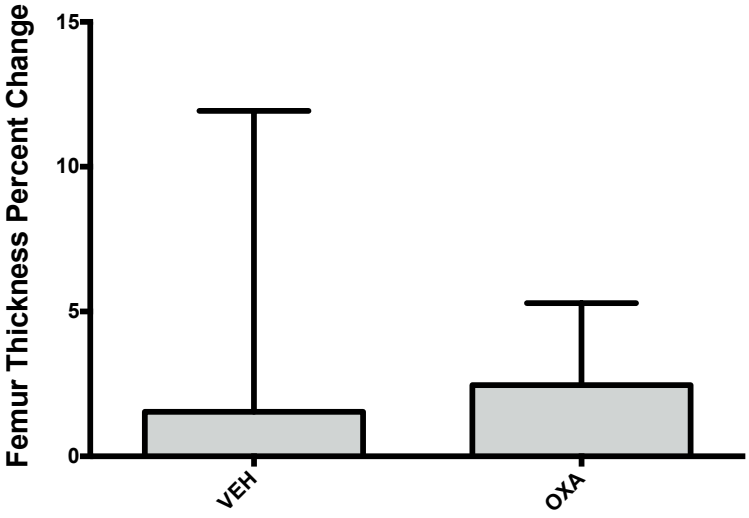

Figure 4B

Figure 4. Femur Measurements. Femur thickness was measured on days 0 and 6 using Vernier calipers. Vehicle (VEH) rats (n=19) received 5% PBS in acetone. Oxazolone (OXA) rats (n=13) received 5% OXA in acetone. Data are means ± SD. A) Data were analyzed by ANOVA followed by Tukey-Kramer post hoc test. B) Data were analyzed by Student's t-test.

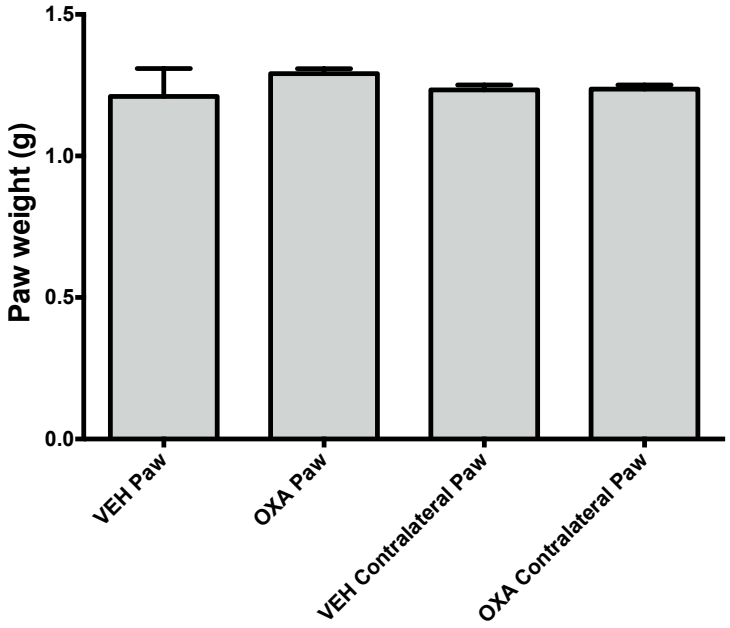

Figure 5. Paw Weight. Paw weight was measured on day 6. Rats were euthanized at day 6 and bilateral paws were removed and weighed. Vehicle (VEH) rats (n=19) received 5% PBS in acetone. Oxazolone (OXA) rats (n=13) received 5% OXA in acetone. Data are means ± SD. Data were analyzed by one-way ANOVA followed by Tukey-Kramer post hoc test.

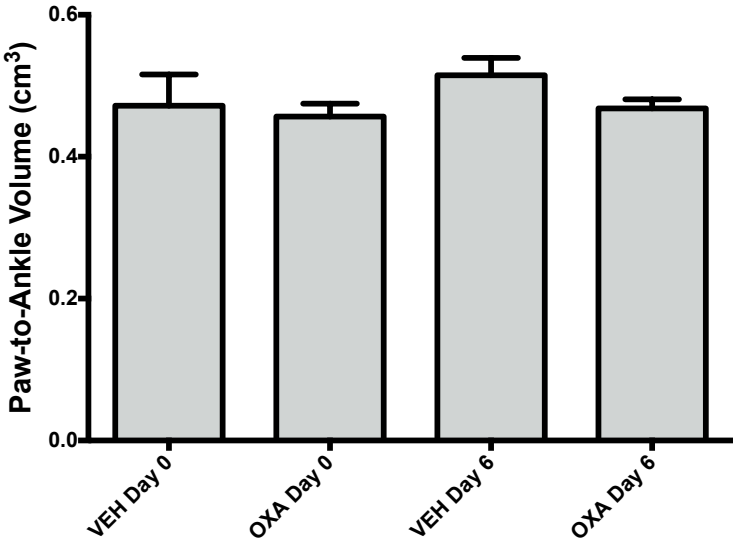

Figure 6A

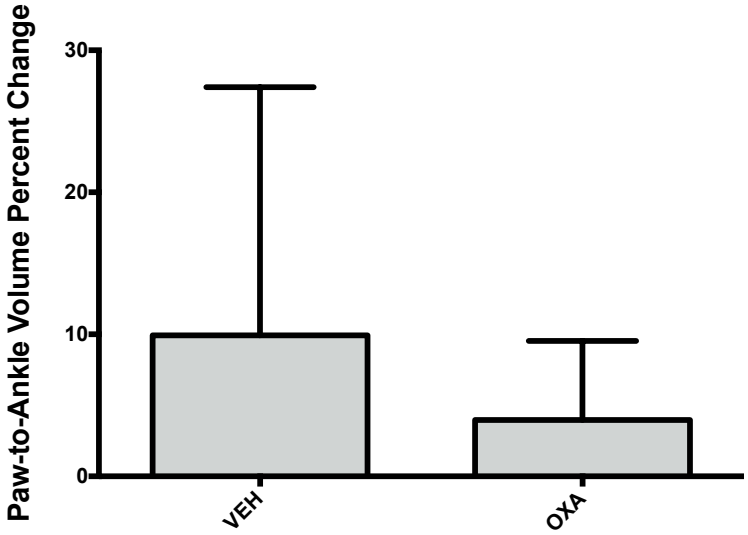

Figure 6B

Figure 6. Paw-to-Ankle Volume. Paw-to-Ankle volume was measured on days 0 and 6 using Vernier calipers followed by truncated cone formula calculation. Vehicle (VEH) rats (n=8) received 5% PBS in acetone. Oxazolone (OXA) rats (n=9) received 5% OXA in acetone. Data are means ± SD. A) Data were analyzed by ANOVA followed by Tukey-Kramer post hoc test. B) Data were analyzed by Student's t-test.

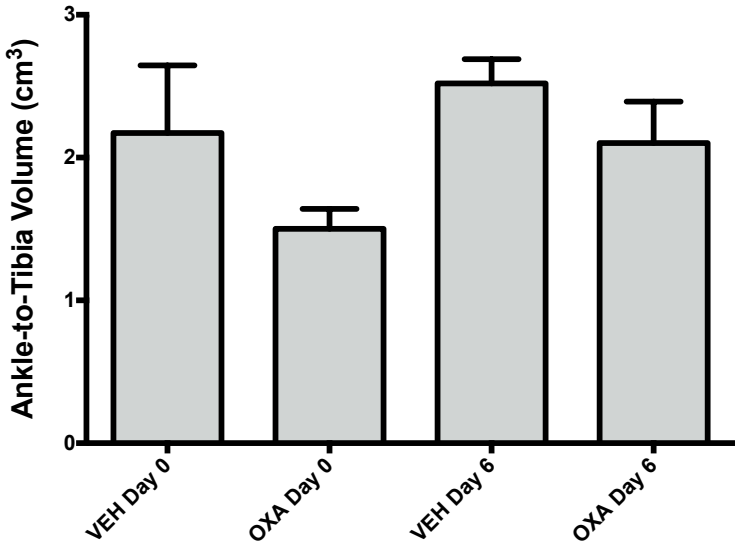

Figure 7A

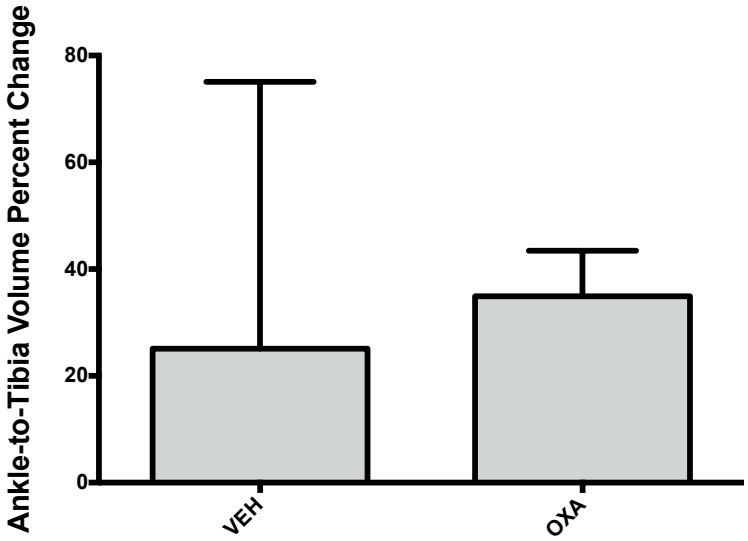

Figure 7B

Figure 7. Ankle-to-Tibia Volume. Ankle-to-Tibia volume was measured on days 0 and 6 using Vernier calipers followed by truncated cone formula calculation. Vehicle (VEH) rats (n=8) received 5% PBS in acetone. Oxazolone (OXA) rats (n=9) received 5% OXA in acetone. Data are means ± SD. A) Data were analyzed by ANOVA followed by Tukey-Kramer post hoc test. B) Data were analyzed by Student's t-test.

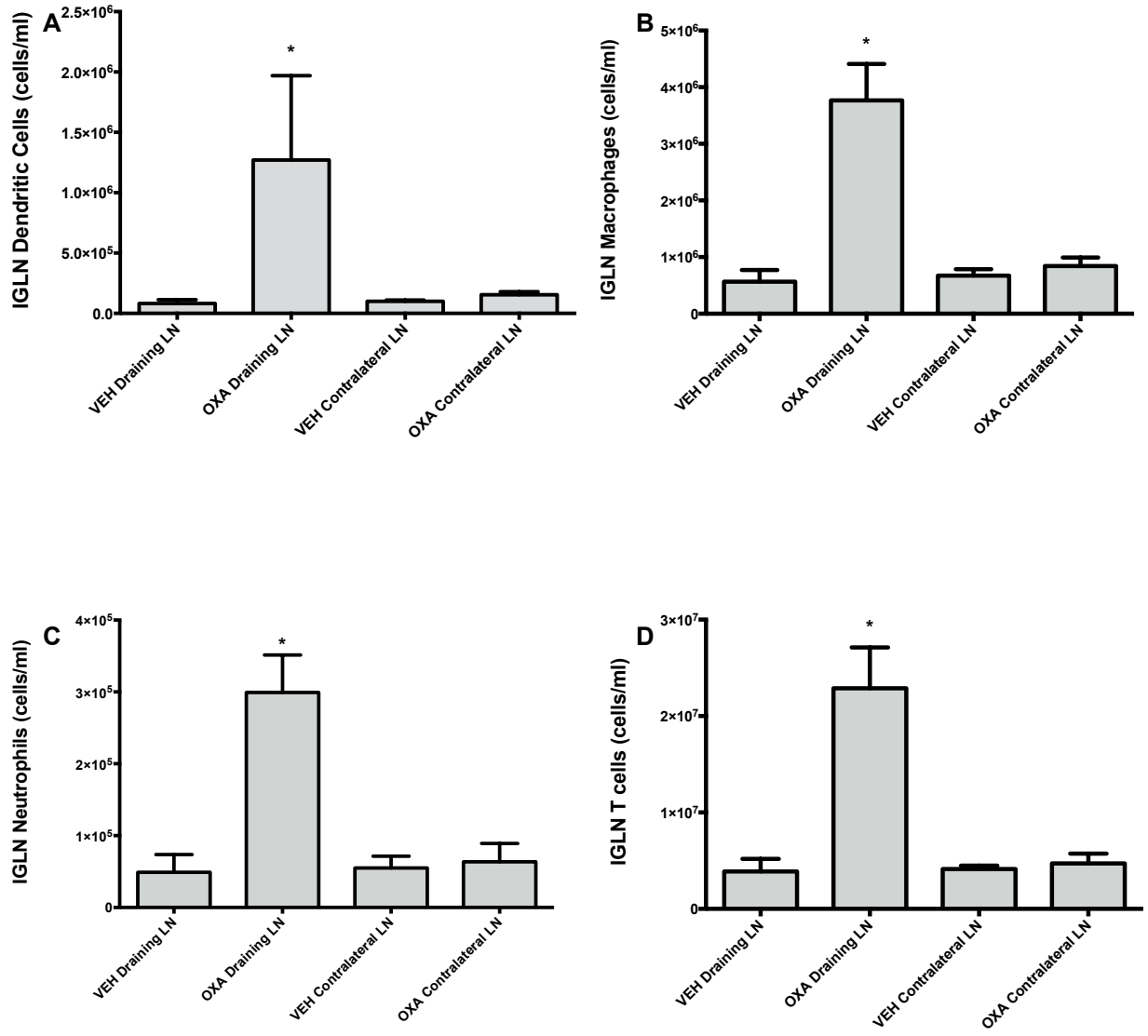

Figure 8. Leukocyte Populations in the Inguinal Lymph Nodes. Rats were euthanized at day 6 and the total leukocyte numbers in the lymph nodes were enumerated using trypan blue exclusion. The individual cell populations were measured by flow cytometry. A) dendritic cells, B) macrophages, C) neutrophils, and D) T cells. Data are means ± SD. Data were analyzed by ANOVA followed by Tukey-Kramer post hoc test. N = 5-12 rats per group. *Significant ($p<0.05$) difference compared to VEH Draining LN and VEH and OXA Contralateral LN.

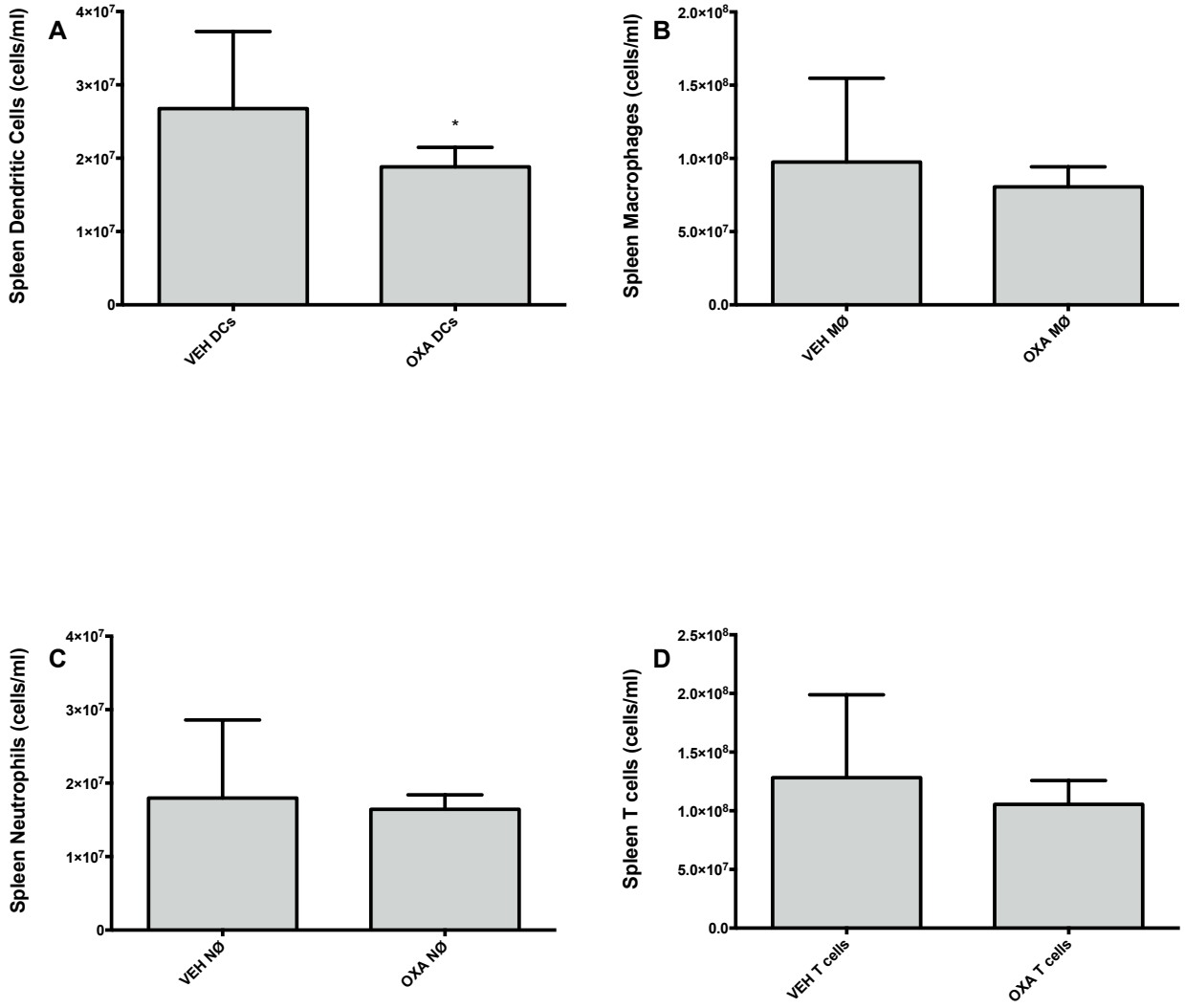

Figure 9. Leukocyte Populations in the Spleen. Rats were euthanized at day 6 and the total leukocyte numbers in the spleen were enumerated using trypan blue exclusion. The individual cell populations were measured by flow cytometry. A) dendritic cells, B) macrophages (MØ) , C) neutrophils (NØ), and D) T cells. Data are means ± SD. Data were analyzed by Student's t-test. *Significantly ($p<0.05$) decreased compared to VEH DCs.

Table 1. Percent Leukocyte populations in the draining inguinal lymph nodes.

	VEH (%)	OXA (%)
Dendritic Cells	1.29 ± 0.67	2.96 ± 1.19*
Macrophages	8.32 ± 3.12	8.92 ± 3.36
Neutrophils	0.70 ± 0.37	0.96 ± 0.69
T cells	55.86 ± 15.76	54.78 ± 16.15

Rats were euthanized at day 6 and the total leukocyte numbers in the draining inguinal lymph nodes were enumerated using trypan blue exclusion. The leukocyte populations in the lymph nodes draining the VEH or OXA treated skin were measured by flow cytometry. Vehicle (VEH) rats received 5% PBS in acetone. Oxazolone (OXA) rats received 5% OXA in acetone. Data are means ± SD. Data were analyzed by Student's t-test. N = 6-12 rats per group. *Significantly ($p<0.05$) increased compared to VEH.

Table 2. Percent Leukocyte populations in the Spleen.

	VEH (%)	OXA (%)
Dendritic Cells	7.52 ± 1.27	6.21 ± 1.25*
Macrophages	22.05 ± 2.81	24.28 ± 3.98
Neutrophils	3.93 ± 1.15	5.84 ± 1.76*
T cells	32.32 ± 3.29	32.13 ± 3.26

Rats were euthanized at day 6 and the total leukocyte numbers in the spleen were enumerated using trypan blue exclusion. The leukocyte populations were measured by flow cytometry. Vehicle (VEH) rats (n=19) received 5% PBS in acetone. Oxazolone (OXA) rats (n=13) received 5% OXA in acetone. Data are means ± SD. Data were analyzed by Student's t-test.
*Significantly ($p<0.05$) different compared to VEH.