Abstract

Purpose: Osteopathic manipulation techniques (OMT) have been utilized by osteopathic physicians to treat a variety of conditions including musculoskeletal dysfunctions, edema, inflammation, and disease. However, the mechanisms by which OMT aides the body in healing are not well understood. The long-term goal of our research is to advance our understanding of the impact of OMT during inflammatory disease, such as sepsis. The purpose of this study was to develop a swine model and to establish surgical techniques that will be used in future studies investigating the impact of OMT on the lymphatic system during disease. We hypothesized that the abdominal lymphatic pump technique (LPT) would enhance thoracic duct lymph (TDL) flow. **Methods:** Four swine subjects (two male and two female) were placed under anesthesia. The thoracic duct was exposed via thoracotomy then cannulated using an angio-catheter. TDL was collected from the four pigs during four-minutes of baseline, four-minutes of LPT, and four-minutes post-LPT. TDL flow was measured by timed collection during each condition. TDL was centrifuged to remove the cellular components, and the supernatant was stored for biomarker analysis. **Results:** TDL flow at baseline was 2.2 ± 1.0 mL/min and LPT increased lymph flow rate to 5.58 ± 1.8 mL/min. In two experiments, thoracic lymph nodes and thoracic duct lymph were collected, and leukocyte were isolated to optimize the flow cytometry staining protocol. **Conclusion:** In our pilot study, LPT increased TDL flow approximately 2-fold in our swine subjects and demonstrated surgical feasibility. In future studies we will study the physiological effects of OMT, including LPT, during sepsis. This knowledge would provide an evidence-based foundation for the use, or contraindication, of OMT during sepsis and aid osteopathic physicians during their therapeutic decision making.

The Effects of Osteopathic Manipulation Techniques on the Lymphatic System

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The Effects of Osteopathic Manipulation Techniques on the Lymphatic System

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Chapter 1: Background and Literature

The Lymphatic System

The lymphatic system's role in the body is to regulate the amount of interstitial fluid in the tissues. The continuous drainage and circulation of fluid from the tissues informs the immune system about the tissue environment which is a crucial component of the adaptive immune response. Lymphatic system obstruction has been identified as a contributing factor in the pathogenesis of cardiovascular disease, inflammation, infectious disease, autoimmune disease, lymphedema, and edema [Adamczyk et al., 2016; Chakraborty et al., 2010]. The lymphatic system has two important mechanisms that facilitate lymph circulation, the intrinsic and extrinsic pumps. The intrinsic pump relies on spontaneous contractions generated in lymphangions [Zawieja, 2009]. Consequently, the extrinsic pump utilizes tissue deformation to circulate lymph within the vessels [Zawieja, 2009]. The presence of endothelial valves ensures unidirectional lymph flow.

Lymph formation begins when blood plasma "leaks" into interstitial tissue from capillary beds. Distinct hydraulic pressure differences between capillary blood and interstitial fluid drives plasma-derived fluid into the interstitium while opposing oncotic pressure differences resulting from greater protein concentration in the plasma regulates this drainage in healthy individuals [Moore Jr. & Bertram 2018]. This tightly controlled balance between hydraulic and oncotic pressure becomes compromised when tissue experiences infection, inflammation, edema, and disease. The initial lymphatic vessels consist of overlapping endothelial cells and surrounding anchoring filaments connected to the extracellular tissue matrix. Fibrous filaments

prevent collapse of the initial lymphatics and allow the vessels to change shape and volume in response to tissue movement [Seffinger, 2018].

The second type of lymphatic vessel, the collecting vessels, are subdivided into short segmented lymphangions by one-way valves and contain smooth muscle capable of generating contractions. Lymph propulsion requires coordinated contraction waves over the length of lymphangions in addition to robust spontaneous contractions promotes unidirectional lymph flow and eventual return to the subclavian veins. The presence of anomalies that distort normal lymphatic anatomy and function can result in loss of tissue fluid homeostasis, impairment in immune traffic, and/or disturbance in lipid and protein reabsorption for the lumen [Rockson, 2010].

Diseases of the Lymphatic System

Recent investigations into the effects of both acquired and congenital lymphatic abnormalities have discovered links to patients with cardiovascular, gastrointestinal, and lymphatic disease. Lymphangiomatosis is a lymphatic disorder characterized by multiple lymphangions with abnormal lymphatic cystic structures thought to be a result of congenital defects in the formation of the lymphatic system [Rasmussen et al., 2015]. Lymphangiomas compromise proper lymphatic drainage and circulation which results in severe lung, heart, joint, and gastrointestinal complications. Chylothorax, or an accumulation of lymph fluid in the thorax, is also a common lymphatic issue seen in ICU patients who have recently undergone cardiothoracic surgery and has been reported to have increased in-hospital mortality, as well as increased length of stay [Burke & Sanjeev, 2018]. Advances in lymphangiography have allowed

the diagnosis of two additional complex lymphatic disease, pulmonary lymphatic perfusion syndrome (PLPS) and central lymphatic flow disorder (CLFD). PLPS is marked by centrifugal flow from the thoracic duct toward peribronchial vessels and lung parenchyma, a reversal of the typical centripetal flow from the periphery towards the central duct [Burke & Sanjeev, 2018]. Meanwhile characteristics of CLFD include reduced or absent central lymphatic flow, effusions in multiple compartments, and dermal reflux of lymph through collateral vessels in the abdominal wall which could be related to thoracic duct outlet obstruction [Burke & Sanjeev, 2018]. The identification of these complex diseases and their wide range of systemic effects has led to more investigations into the lymphatic system's role in chronic and acute diseases.

Several pathological conditions including autoimmunity, microbial infection and chronic allograft rejection lead to chronic inflammation which has been shown to cause remodeling of the lymphatic network [Liao & Yves von der Weid, 2014]. This remodeling is characterized by growth of the initial lymphatics, a process known as lymphangiogenesis and may represent an attempt to compensate for impaired lymphatic drainage in order to maintain clearance of interstitial fluid [Liao & Yves von der Weid, 2014]. Inflammation also impairs lymphatic contractile ability by increasing the vessel diameter and decreasing contraction frequency resulting in poor circulation and compromised immune function. Lymphatic valves that normally ensure one directional flow become overwhelmed by the increased fluid accumulation which can manifest into additional complications like lymphedema.

Failure of the development of the lymphatic system leading to either structural or functional abnormalities that impair maintenance of interstitial fluid balance results in primary lymphedema [Adamczyk et al., 2016]. Researchers have currently identified nine causal genes,

with the goal that identification of new genes will increase our understanding of the aetiopathogenis of lymphatic disease [Adamczyk et al., 2016]. The most common form of lymphedema is secondary lymphedema which affects over 250 million people worldwide. In the United States, secondary lymphedema is the most diagnosed lymphatic disease with most cases resulting from mastectomy, congestive heart failure, or reconstructive surgery [Zawieja, 2009]. Unfortunately, there is no cure for lymphedema, but successful management of the disease can be achieved with exercise, compression garments, and manual lymphatic drainage.

Physical Medicine Treatment for Lymphatic Disease

Secondary lymphedema is most prevalent lymphatic disease in the United States with most patients reporting significant negative effects involving function, comfort, and quality of life. Complex (or complete) decongestive lymphatic therapy (CDT) is a two-stage treatment program focused on improving patients' range of motion in addition to decreasing limb volume and infection probability. The four components of CDT are manual lymphatic drainage (MLD), compression therapy, lymph-reducing exercises and skin care [Ezzo et al., 2016]. The first phase of CDT, which lasts two to four weeks, consists of specific MLD, range of motion exercises, and compression with multilayered compression bandages [Gradalski et al., 2015]. Once the limb is sufficiently reduced in volume, Phase 2 is initiated with the goal of maintaining volume reduction using a custom fitted compression garment or sleeve [Gradalski et al., 2015; Ezzo et al., 2015].

MLD is a specific hands-on therapy performed by licensed therapist trained extensively in anatomy and physiology of the lymphatic system. MLD is designed to reduce lymph swelling

by enhancing lymphatic drainage [Ezzo et al., 2016]. The proposed mechanism of MLD is to enhance interstitial drainage and filling of the primary lymphatic vessels which facilitates dilation and contraction of the lymphatic vasculature [Gradalski et al., 2015]. The first techniques of MLD are deep breathing and massage of the unaffected areas of the body to remove blockages in lymphatic vessels and stimulate lymph flow. Then, the proximal end of the affected area is continuously massaged in an upward direction, gradually moving towards the distal end. By utilizing both skin stretching and gentle compression, MLD is also believed to reroute lymph from damaged lymphatic vessels to viable ones. Systematic reviews examining MLD effectiveness present contraindicatory reports from no benefit [Haung et al., 2013], to small benefit [McNeely et al., 2004], to substantial benefit [Moseley & Piller, 2006], to inconclusive benefit [Devoogdt et al., 2010].

Pneumatic compression pumps are another form of compression therapy used to treat lymphatic disease. The standard practice consists of an arm or leg sleeve that fills with air when hooked to an electric pump device. The compression pump is comprised of gradual pressure gradients that help lymph flow through the lymph vessels [Uzkeser et al., 2015]. Lymphedema treatment studies report pneumatic pumps achieved a reduction of limb girth measurement by 37% and up to 86.6% [Lachmann & Tunkel, 1992; Richmand et al., 1985]. Contraindications for this therapy include patients with renal disease, congestive heart failure, pulmonary edema, acute skin infections, and acute deep vein thrombosis. When treating post-oncological lymphatic disease, patients should also be free of metastasis of the limb to prevent the risk of spreading the malignancy [Klein et al., 1978].

Osteopathic Manipulation Techniques

Therapies have designed to promote lymph circulation and to restore normal function of the lymphatic system. The osteopathic medical field has long recognized the importance of homeostasis and the interrelationships of body systems [Degenhardt, 1996]. Osteopathic physicians prescribe osteopathic manipulation as treatment for musculoskeletal systemic pathophysiologic problems in addition to general health maintenance and enhancement strategies [Seffinger, 2018]. Since its inception by Andrew Taylor Still, one of the primary goals of osteopathic treatment is improving lymph drainage [Seffinger, 2018]. Many OMT are designed to enhance lymph flow and remove substances blocking the lymphatic vessels [Degenhardt, 1996]. These OMT are thought to influence lymph flow directionality by promoting continuous rhythmic contractions of the vessels and surrounding musculature.

Specifically, myofascial release techniques utilize stretching and soft tissue compression to treat a variety of musculoskeletal, lymphatic, and circulatory dysfunctions in the body. This soft tissue technique is thought to improve lymph flow by directly applying external pressure to tissues to aid the removal of blockages within the lymph vessels that limit venous and lymphatic return. Osteopathic physicians have historically used lymphatic pump techniques (LPT) to treat infection and edema. LPT proposed mechanism of action affects every phase of lymphatic circulation beginning with lymph formation by applying direct motion against the tissue; the vascular phase in which lymph is circulated using systemic and rhythmic pumping; and the terminal phase by encouraging additional pressure changes in the intra-abdominal and intrathoracic cavities to increase return of lymph to venous circulation [Seffinger, 2018]. While

clinical evidence supports the use of OMT to treat disease, the physiological mechanisms are still not well understood.

LPT are clinically applied to the spleen, liver, thoracic cage, or feet [Seffinger, 2018] (See Figure 1). LPT were designed to facilitate fluid movement and enhance immunity in patients with disease [Franzini et al., 2018]. LPT are used to treat patients with congestive heart failure, upper and lower gastrointestinal tract dysfunction, respiratory tract infection, and edema [Seffinger, 2018]. One clinical study discovered that patients treated with thoracic lymphatic pump after cholecystectomy recovered quicker and returned to baseline forced vital capacity earlier than patients only treated with incentive spirometry [Sleszynski & Kelso, 1993]. Animal studies have found LPT, in addition to levofloxacin treatment, significantly reduced colony forming units of *Streptococcus pneumoniae* compared to levofloxacin plus sham treatment in rats [Hodge et al., 2015]. While a mainstay in osteopathic clinical practice, questions remain about the mechanism(s) responsible for OMT's therapeutic benefits.

Animal Studies

Animal studies utilizing OMT, massage, limb rotation, tissue compression, and exercise have provided substantial insight into the mechanisms by which manual manipulation aide in tissue health by promoting lymph flow. Specifically, studies identified lymphatic flow enhancement in rats [Huff et al., 2010; Takeno et al., 2013], rabbits [Ikomi & Ohhashi, 2000], dogs [Knott et al., 2005; Hodge et al., 2007; Downey et al., 2008; Hodge et al., 2010; Prajapati et al., 2010; Schander et al., 2012; Schander et al., 2013] and sheep [McGeown et al., 1988]. A series of experiments demonstrated enhanced thoracic duct lymph flow, first in response to lymphatic pump techniques and treadmill exercise in conscious dogs [Knott et al., 2005], then in

a lymphedema model, executed by inferior venal caval restriction, which saw similar results in response to LPT administration [Prajapati et al., 2010]. Additional studies have supported the use of LPT to enhance lymph flow in both dogs and rats [Hodge et al., 2007; Huff et al., 2010]. In 2007, Hodge and colleagues also demonstrated that LPT significantly enhanced lymph leukocyte flux and thoracic duct lymph flow in dogs thereby enhancing immunological function [Hodge et al., 2007]. Studies have also demonstrated increased leukocyte concentrations in thoracic and mesenteric lymph [Hodge et al., 2010] and increased circulation of inflammatory mediators originating from the mesentery [Hodge et al., 2010; Schander et al., 2012]. The most recent study demonstrated the reduction of inflammatory response by macrophages *in vitro* after addition of lymph fluid [Castillo et al., 2018]. These final studies provide evidence of LPT's effect on the lymphatics and immune system, therefore supporting the use of OMT in clinical settings for medical treatment.

In 2010 Hodge and colleagues conducted a study investigating LPTs ability to mobilize leukocytes for gut-associated lymphoid tissue (GALT) into lymph. The investigators inserted catheters into either the thoracic or mesenteric lymph ducts of doges, then fluorescently labeled mesenteric lymph nodes (MLN) *in situ*. Lymph was collected during four minutes of pre-LPT, four minutes of LPT, and ten minutes after the cessation of LPT. LPT significantly increased lymph flow and leukocytes in both mesenteric and thoracic duct lymph [Hodge et al., 2010]. LPT also significantly increased the mobilization of leukocytes from MLN into thoracic duct lymph [Hodge et al., 2010]. While this study found that the mesenteric-derived leukocytes remained statistically elevated throughout the LPT treatment, a decline of leukocytes was observed during the last 3-4 minutes of LPT. This finding suggests that LPT mobilizes

mesenteric lymph from a fluid pool that depletes within a few minutes of treatment [Hodge et al., 2010].

Schander and colleagues investigated the lymphatic system's response to repeated applications of LPT. They discovered that LPT repeatedly enhanced TDL flow, TDL leukocytes, leukocyte flux, and flux of cytokines and chemokines. The second LPT treatment was administered two hours after the first, demonstrating that LPT mobilizes lymph from a reservoir that is replenished by two hours [Schander et al., 2013]. This study also reported that LPT increased lymphatic flux of cytokines, keratinocyte-derived chemoattractant (KC), superoxide dismutase (SOD) and nitrite (NO_2^-) [Schander et al., 2013]. Ultimately Schander provided insight into the lymphatic system's response to manual stimulation and provided clinical support for repeated treatments of LPT to mobilize leukocytes and other inflammatory mediators into lymphatic circulation.

In 2018, Castillo and colleagues were the first to demonstrate that TDL suppresses macrophage activity in vitro. The results of this study suggest that thoracic duct lymph contains bioactive molecules capable of mitigating the inflammatory response. TDL collected before LPT, during LPT, and after LPT equally suppressed activity of macrophages [Castillo et al., 2018]. This study was consistent with other reports that LPT increased TDL flow and protein flux [Castillo et al., 2018; Hodge et al., 2007; Hodge et al., 2010; Schander et al., 2012; Schander et al., 2013]. In conclusion, the results of this study were crucial to our understanding of the lymphatic system's relationship with immune function and the first to provide scientific evidence for the clinical use of LPT to enhance both the lymphatic and immune systems.

Sepsis

Sepsis is a syndrome defined by life-threatening organ dysfunction that develops from a dysregulation host response to infection [Cecconi et al., 2018]. This unusual systematic reaction is characterized by a hyperinflammatory response followed by an immunosuppressive phase during which multiple organ dysfunction is present [Faix, 2013]. The cause of multiple organ failure during sepsis remains unknown, but cognitive impairment, lung, liver, and/or kidney injury are the most common complications in patients diagnosed with severe sepsis. Each year 1.7 million Americans develop sepsis and approximately 270,00 die as a result. Elderly and immunosuppressed individuals have a higher incidence of sepsis, as well as a higher mortality rate, suggesting that pre-existing immune dysfunction is a major risk factor for sepsis [Faix, 2013].

Septic shock, described as a clinically subset of sepsis cases in which a patient does not respond to adequate fluid resuscitation or vasopressor therapy, is the most common terminal event of severe sepsis [Faix, 2013; Cecconi et al., 2018]. Early identification and treatment are crucial to short- and long-term survival. Pro-inflammatory cytokines and chemokines, proteins, and markers of neutrophil and monocyte activation have been identified as biomarkers that enhance the inflammatory response during sepsis [Faix, 2013]. Additionally, elevated lactate concentration is an important prognostic measurement in septic patients and reduction concentration is associated with improved outcomes [Simmons & Pittet, 2015]. Treatment for sepsis includes initial fluid resuscitation, antibiotic therapy, removal of infected tissue or devices, and vasoactive drugs. Recent data suggests that mortality due to sepsis has dropped

substantially over the last two decades, but still remains one of the world's leading causes of death.

A prominent area of research has just begun to define the role of the lymphatic system in the development and progression of sepsis. When the gut is exposed to excessive or prolonged splanchnic hypoperfusion during traumatic injury, it releases nonbacterial gut-derived inflammatory and tissue injurious factors that contribute to systemic inflammatory response syndrome (SIRS), acute respiratory distress syndrome (ARDS), and multiorgan dysfunction syndrome (MODS) [Deitch, 2010]. Researchers hypothesize that the intestinal lymphatics are the route for gut-derived pathogens and proinflammatory non-microbial materials to enter circulation and promote the inflammatory response which eventually results in deleterious functional and structural organ damage. The mesenteric lymph is first exposed to the pulmonary vasculature, which is also the first and most commonly injured organ in critically ill patients through development of ARDS and the subsequent development of MODS [Assimakopoulos et al., 2018; Deitch, 2010].

Several major experimental observations support the gut lymph hypothesis. First, ligation of the major intestinal lymph duct prevents the development of early ARDS and MODS by preventing lymph from entering systemic circulation [Deitch, 2010]. Secondly, neutrophil activation, cardiomyocyte and endothelial cell injury as well as red blood cell dysfunction was observed when mesenteric lymph from shocked, but not sham-shocked, animals was administered during in vitro studies [Deitch et al., 2006]. Additional research is required to understand the pathophysiological mechanisms of the gut that promote sepsis in order for therapy development to achieve optimal effect.

The long-term goal of this research is to define the role of the lymphatic system and the impact of lymph enhancing therapies, such as OMT, during sepsis. The results from this study will expand our basic understanding of the lymphatic and immune system's response to OMT and establish a large animal model that will be used in future studies investigating sepsis. This research has the potential to help guide practitioners in their medical practice and develop clinical research which will greatly improve healthcare.

Chapter 2: Research Project

I. Specific Aim: Quantify the effect of OMT on thoracic duct lymph flow in domestic swine.

We hypothesized that abdominal LPT would increase lymphatic flow in the thoracic duct of domestic swine. TDL flow was measured by timed collection and TDL was collected before, during, and after each OMT. Comparisons were made before, during, and after LPT.

II. Significance & Innovation

This study is significant because it is the first to determine if the abdominal lymphatic pump will stimulate lymphatic flow in a pig model. The proposed approach allowed us to collect data that could not be obtained in human subjects and further strengthened our understanding of OMT's effect on the lymphatic and immune systems. The development of this large animal model is essential for the continuation of mechanistic studies seeking to define the mechanism(s) responsible for OMT's health benefits. Importantly, identifying the physiological effects of OMT on the lymphatic and immune systems will aid osteopathic physicians in their clinical practice and guide future clinical research.

III. Materials and Methods

Animals

Four swine subjects were used in this study and were housed and fed according to the Institutional Animal Care and Utilization Committee (IACUC) in the University of North Texas Health Science Center animal facility. This study was approved and conducted in accordance with IACUC protocol number, 2020-009. This investigation was conducted in both male and female domestic pigs for several reasons. Of the common research animals, pigs are the most physiologically similar to humans in terms of heart rate, blood pressure and alveolar ventilation. The thoracic duct's anatomical location and course is well-documented in pigs [Chanoit et at., 2007], and has proven consistent across animals, and the pig's size permits collection of multiple lymph samples in a statistically robust, repeated-measures experimental design. Four domestic Yorkshire cross pigs (two male and two female) were used for this pilot investigation. Pig #1 was a castrated male that weighed 99.0 lbs. (44.91 kg). Pig #2 was female that weighed 130.0 lbs. (58.97 kg). Pig #3 was female that also weighed 130.0 lbs. (58.97 kg). Pig #4 was a castrated male that weighed 160 lbs. (72.57 kg).

Surgical Procedure

The first two pigs were fed a high fat diet 24 h prior to surgery to enhance the visualization of the lymphatic vessels. During the first two surgeries, the high fat

diet was given one day prior to surgery, but after careful consideration our team decided to increase the feeding time to five days prior to surgery for the remaining two surgeries. The absorption of lipid from the alimentary tract and subsequent lipid uptake by the mesenteric lymphatics imparts a distinct, milk-white appearance to the thoracic duct lymph [Yen & Davies, 2016]. Fifteen hours prior to surgery, the pigs were fasted. On the day of surgery, the pigs were sedated with a cocktail of telazol (5 mg/kg IM) and xylazine (5 mg/kg IM) and intubated with a cuffed endotracheal tube. The pig was carefully placed in the prone position on a circulating water heating pad. A rectal probe was placed to monitor body temperature. A surgical plane of anesthesia was maintained by mechanical ventilation (tidal volume 12-15 ml · kg^{-1}; 12-14 cycles/min) with 1-4.5% isoflurane in 100% O_2. Arterial pH was maintained at 7.35-7.45 by adjusting ventilation or by administering $NaHCO_3$ via the femoral vein. Epidermal electrodes were placed for standard limb lead II electrocardiography. The right femoral artery and vein was exposed by inguinal incision and blunt dissection. A 7 Fr polyurethane catheter was inserted into a branch of the femoral artery and advanced into the abdominal aorta to monitor systemic arterial blood pressure and sample arterial blood. Another catheter was inserted into a branch of the femoral vein for infusing medications and 0.9% NaCl. Next, the pig was carefully repositioned in left lateral recumbency, and a right thoracotomy was performed in the 3-4^{th} intercostal space, at which point positive pressure ventilation was initiated at an end-respiratory pressure of 5 cm H_2O. We chose thoracotomy as our approach to access the thoracic duct because

OMT was applied to the abdomen and we did not want to contact or apply pressure to the surgical site. The ribs were retracted, and the thoracic duct, which courses alongside the thoracic aorta [Chanoit et al., 2007; Yen, 2016], was accessed via a mediastinal incision. An incision was made in the rostral duct segment, and a 5 Fr polyurethan cannula was inserted into the duct through an incision, advanced 3-4 cm in the caudal direction, and secured with suture. The thoracic duct was cannulated as described above. Lymph was collected in Ethylenediaminetetraacetic acid (EDTA) coated tubes. Lymph samples were collected during four minutes of baseline, four minutes abdominal LPT, and four minutes post-LPT. Comparisons were made between baseline, LPT and post-LPT.

Throughout the surgery, oxygen levels, temperature, pulse, tidal volume, and oxygen saturation level were monitored to ensure the swine subjects were tolerating the surgical procedure and LPT application.

Osteopathic Manipulative Techniques

The pig remained in lateral recumbency for the OMT. The abdominal lymphatic pump technique was chosen for the study. Kendi Hensel, D.O., Ph.D. and Ryan Seals, D.O. developed the OMT that was applied.

The abdominal LPT was adapted for dogs by Hollis H. King, D.O., Ph.D. and Artur Schander, D.O., Ph.D. [Hodge et al., 2007; Hodge et al., 2010; Schander et al., 2012; Schander et al., 2013]. LPT was applied in a similar manner to pigs. During LPT, the administrator contacted the ventral side of the pig's abdomen with his/her hands

placed bilaterally below the costo-diaphragmatic junction (See Figure 2). Pressure was exerted medially and cranially to compress the abdomen until resistance is encountered against the diaphragm, and then pressure was released. Abdominal compressions were administered approximately once per second for a total of four min.

Outcome measures

Thoracic duct lymph (TDL) flow was measured by timed collection during 1) baseline, 2) performance of LPT, and 3) for four minutes post-OMT. These intervals were chosen based on published studies from our lab using dogs [Hodge et al., 2007; Hodge et al., 2010; Schander et al., 2012; Schander et al., 2013]. This data is important because it will guide osteopathic practitioners in their application of OMT clinically.

Euthanasia

A pericardiotomy was performed to expose the heart. The two poles of a 9-volt battery were applied to the epicardium to induce ventricular fibrillation under a surgical anesthetic plane. Then, the heart was excised. This method of euthanasia is concordant with the 2020 Edition of the American Veterinary Medical Association *Guidelines for the Euthanasia of Animals.*

Cell Counting

TDL was first centrifuged at 800 rpm for ten minutes at 4^{o}C to isolate leukocytes. Lymph supernatant was removed and stored at -20 ^{o}C future biomarker analysis. Trypan blue exclusion was used to determine the total number of viable leukocytes. The total number of viable leukocytes were counted using a hemocytometer.

Flow Cytometry

Two-color immunofluorescent staining was performed to identify macrophage, T and B cell populations using FITC-labeled mouse anti-pig Monocyte/Granulocyte monoclonal antibody (mAb) (MCA6100F, BioRad, Hercules, CA), FITC-labeled mouse anti-pig CD3 (mAb) (MCA5951F, BioRad) and FITC-mouse anti-pig B cell mAb (MCA60099F, BioRad). A total of 10^{6} cells were incubated with 10ul or 20ul of mAb for 30 min at 4 ^{o}C. The cells were washed in staining buffer [Mg2+-free, Ca2+-free phosphate buffered saline, 1% fetal bovine serum (Hyclone)] and fixed with 0.5% paraformaldehyde until analyzed. The cells were analyzed on a BD LSR II flow cytometer, and data were analyzed using FlowJO software (TreeStar Inc.). Lymphocyte gates and detector voltages were set using unstained (control) cells, and stained cell populations were seen as distinct peaks or clusters of cells. The proportion of each cell population was expressed as the percentage of the number of stained cells.

IV. Results

Surgical Approach

During the first surgery we determined that a left thoracotomy was the best method to locate and cannulate the thoracic duct in swine (See Figures 3 & 4). Our subjects maintained normal vital functions throughout surgery and LPT administration (See Tables 1, 2, 3 & 5). In future studies we recommend keeping freshly collected lymph on rotators and not placing fresh samples on ice because is caused our samples to clump in experiments 1 & 2 which compromised our lymphocyte cell counts.

Experiment 1: Castrated male swine (99 lbs.)

A left thoracotomy was performed, and the thoracic duct was cannulated using an 18' angio-catheter. Lymph was collected during four minutes of baseline and four minutes of abdominal LPT. Baseline flow rate was 2.5 mL/min and increased approximately 2-fold to 5.5 mL/min during abdominal LPT. The catheter began to clot after LPT was stopped, which prevented us from collecting post-LPT samples. We noted in the lab that the lymph samples contained red blood cells and also appeared slightly clotted which resulted in inaccurate cell counts. The animal's vitals remained normal and LPT did not augment vital signs (See Table 1).

Experiment 2: Female swine (131 lbs.)

As in experiment 1, a thoracotomy was performed to expose and cannulate the thoracic duct. While collecting lymph samples, the catheter became clotted, stopping lymph flow. The catheter was cleared, but the flow rate at baseline was greatly reduced, 0.89 mL/min, compared to our previous experiment. Continued clotting and variable flow rates made the collection of lymph for this animal inconsistent therefore this experiment was terminated. In addition, the collected samples were noted to be heavily clotted when analyzed in the lab which prevented us from determining accurate cell counts and flow cytometry data. While there was increased clotting in the catheter for this experiment, the animal's vital signs remained normal throughout the surgery and during LPT administration (see Table 2).

It was decided after the second experiment, that the lymph samples would be collected in EDTA coated tubes, kept at room temperature, and placed on a blood rotor to prevent clotting for the remaining experiments.

Experiment 3: Female swine (130 lbs.)

Consistent with the previous two experiments, a left thoracotomy was performed, and the thoracic duct was cannulated using a 22' angio-catheter. Lymph was collected during four minutes of baseline, four minutes of abdominal LPT, and four minutes post-LPT. Baseline flow rate was 3.1 mL/min and increased approximately 2-fold to 7.5 mL/min during abdominal LPT. Post-LPT flow rate was 5 mL/min. Rotating the samples at room temperature prior to lymph fluid separation reduced clumping in

the EDTA collection tubes. The animal's vitals remained normal and LPT did not augment vital signs (See Table 3). The lymph samples for all conditions had a large number of red blood cells that could not be removed; therefore, we did not use this lymph to determine leukocyte counts or for leukocyte flow cytometry. To optimize our antibody staining protocol for flow cytometry, we collected two thoracic lymph nodes. The lymph nodes were suspended in sterile PBS, homogenized, and washed with wash media [Hodge et al., 2010]. The leukocytes were stained using the manufacturers recommended protocol. We used two volumes of antibodies for staining, 10 ul (recommended by the manufacturer) and 20 ul. In the thoracic lymph nodes, the percentage of granulocytes/monocytes stained with 10 ul or 20 ul of antibodies were similar to what was reported by the manufacture when they stained mesenteric lymph nodes using 10 ul. However, the percentage of B and T cells were lower than expected based on our previous experiments in dogs. There was little difference between the percentage of cells stained with 10 ul or 20 ul of antibody; therefore, we chose 10 ul for future staining protocols (See Table 4).

Experiment 4: Castrated male (160 lbs.)

A left thoracotomy was performed, and the thoracic duct was cannulated. We collected lymph for eight minutes of baseline and two minutes of LPT before the catheter came out of the thoracic duct. The baseline flow rate was 1.0 mL/min and increased approximately 3-fold to 3.75mL/min during abdominal LPT. Following LPT, the catheter had to be reinserted. After re-cannulation the flow rate increased from 2.0

mL/min at baseline 2 to 4.1 mL/min, approximately 2-fold. Post-LPT flow rate remained elevated at 3.5 mL/min. The lymph samples continued to have large amounts red blood cells. We also noted the cell separation protocol was not pelleting the cells and while separating the supernatant, cells were accidently removed in our samples. The animal's vitals remained normal and LPT did not augment vital signs (See Table 5). As in previous experiments, the lymph supernatant was stored for future biomarker analysis to determine if the composition is altered during LPT.

Average TDL Flow Rate:

The means and standard deviations of TDL flow from experiments 1, 3, and 4 are illustrated in Figure 3. TDL flow at baseline was 2.2 ± 1.0 mL/min and LPT increased lymph flow rate to 5.58 ± 1.8 mL/min.

V. Discussion

The most important discovery of our pilot study was that LPT increased TDL flow in swine subjects. This report is the first to quantify the effect of LPT on the thoracic duct flow in a swine model. This data is consistent with our previous studies using dogs [Hodge et al., 2007; Hodge et al., 2010; Schander et al., 2012; Schander et al., 2013; Castillo et al., 2018] and rats [Huff et al., 2010]. By increasing TDL flow, LPT may also release lymphocytes and inflammatory molecules into the circulation of pigs.

Another important change to our protocol that improved our experiments was increasing the length of time feeding our pigs the high fat diet. The third pig's lymph had a markedly increased milky appearance upon visualization of the thoracic duct. This finding was consistent with the fourth pig as well. The fourth pig's thoracic duct also appeared larger with an increased width which improved visualization and ease cannulation. During future investigations of the lymphatic system, we recommend feeding the pigs a high fat diet at least 5 days prior to surgery.

We discovered in experiment 4 that LPT repeatedly enhanced TDL flow. This data is consistent with previous studies performed in canine subjects [Schander et al., 2013]. LPT 1 produced an approximate 3-fold increase in TDL flow from 1 mL/min to 3.75 mL/min compared to LPT 2 increase from 2 mL/min to 4.1 mL/min, an approximate 2-fold increase. This finding suggests that multiple LPT could be applied in a short time frame.

An unexpected, but consistent finding we had during each of our experiments was the presence of red blood cells in our collected lymph samples. After more research on pig lymphatic anatomy, we discovered that this finding was consistent with another experiment by Yen and colleagues in 2015 [Yen et al., 2015]. While the number of RBCs present in the lymph varied in each experiment, the greatest number was observed in the third and fourth experiments. The cause of the observed variation in numbers of RBC still needs to be investigated in addition to the site of entry from the venous or arterial vasculature. In future studies we must refine our protocol to remove the RBCs present in swine thoracic duct lymph. This

will ensure accurate leukocyte counts. A leukocyte gradient may be an option to remove the RBCs from lymph.

Importantly, we demonstrated surgical feasibility and finalized our surgical thoracotomy protocol and lymph collection methods which will enable our lab's continued investigation of the lymphatic system during disease. The development of this robust pig model will guide future studies investigating the physiologic effects of OMT on the lymphatics during sepsis.

Understanding OMT's physiological effects and underlying mechanism is essential to discern when OMT might be detrimental. Specifically, during sepsis the increased delivery of leukocytes and inflammatory mediators in lymph to the systemic circulation is believed to contribute to multiple organ dysfunction [Assimakopoulos et al., 2018; Deitch, 2012]. Acquiring this information is especially important for osteopathic practitioners to provide scientific-based evidence for use, or contraindication, of OMT during their therapeutic decision making.

VI. **Limitations**

We acknowledge that OMT administration in research animals is not equivalent to that of the human population. However, our proposed mechanism of lymph collection cannot be utilized in human subjects, making the data we acquire of important interest. Indirect radionuclide lymphoscintigraphy, which relies on intra- or subcutaneous injection of a large, radiolabeled molecule, is the most common lymphatic imaging technique used confirm lymphedema diagnoses in human

patients [Rockson, 2010]. Although this technique is extremely useful clinically, it would not allow us to directly measure TDL flow or the concentration of TDL lymph. Lymphoscintigraphy can be used to identify smaller lymphatic vessels for investigation in future studies and/or investigate anatomical anomalies in diseased pigs. Our study seeks to provide empirical evidence for the clinical use of LPT.

We discovered that LPT increased TDL flow by approximately two-fold. We acknowledge that four animals are a small number of subjects, and our lab has future experiments planned that will continue investigating TDL flow rate in response to OMT. Specifically, we will quantify changes in leukocyte concentrations and populations, cytokines, chemokines between baseline, LPT, and post-LPT.

VII. Future Directions

OMT's biologic activity and mechanisms that support enhanced protection against disease are still not well understood. Redistributing inflammatory mediators and leukocytes by increasing lymph output and circulation using OMT therapies could provide protection against inflammatory and infectious disease. LPT-mobilized lymph contains cytokines and chemokines [Schander et al., 2012; Schander et al., 2013] and can suppress the macrophage mediated inflammatory response *in vitro* [Castillo et al., 2018]. Therefore, increasing the circulation of inflammatory mediators can enhance the inflammatory response to pathogens. The biologic activity of TDL collected will be defined in future studies. Furthermore, we can use biologic assays and high-performance liquid chromatography to identify differences

in proteins and molecules in TDL collected during baseline, LPT, and post-LPT. These projected studies could identify the bioactive factor present in TDL.

This study investigated one OMT technique, abdominal LPT. Future studies are needed to investigate other prominent OMT techniques including myofascial release, thoracic LPT, and pedal pump and their effects on the lymphatic system. This knowledge would increase our understanding of the lymphatic system's reaction to a variety of techniques and further strengthen our understanding of the pathophysiological mechanisms of OMT.

Myofascial release can be applied to the thoracic inlet region to remove any tissue restrictions around the thoracic duct, diaphragm, and intestinal mesentery. This technique is applied by contacting the thoracic outlet or abdominal region with the physician's palms, then gently applying pressure until the tissue softens and tension is normalized.

Pedal pump is applied to create an oscillatory force to mobilize fluid in the lower extremity and the whole body. The physician should stand at the caudad region of the animal and contact the hind limbs bilaterally by grasping each limb with one hand. The limbs are then used as levers to create a rhythmic oscillatory force.

The thoracic pump technique can be investigated in future studies with the cannulation of the cisterna chili. Future investigators can measure the impact of thoracic LPT on the lymphatics by collecting lymph from the cisterna chili while thoracic LPT is administered to the subject's thoracic cage.

Additionally, the development of this pig model will guide future studies in their investigation of the effects of OMT on the lymphatic system in diseased models. Current swine disease models include sepsis, metabolic syndrome, ischemic/reperfusion injury, and cardiovascular disease. Sepsis is a syndrome defined by life-threatening organ dysfunction that develops from a dysregulated host response to infection [Ceconni et al., 2018]. This unusual systematic reaction is characterized by a hyperinflammatory response followed by an immunosuppressive phase during which multiple organ dysfunction is present [Faix, 2013]. Pro-inflammatory cytokines and chemokines, proteins, and markers of neutrophil and monocyte activation have been identified as biomarkers that enhance the inflammatory response during sepsis [Faix, 2013]. Furthermore, studies have identified gut lymphatics as the primary route for biologic factors leaving the gut that induce sepsis and multiple organ dysfunction following trauma [Deitch, 2010], which raises the question as to whether LPT or other forms of abdominal OMT should be used under these conditions. The development of this robust pig model will guide future studies in the investigation of OMT on the lymphatics during sepsis. Acquiring this information is especially important for osteopathic practitioners to provide scientific-based evidence for use, or contraindication, of OMT during sepsis.

In conclusion, studies like this one are essential for identifying the underlying mechanisms responsible for the health benefits of OMT and thus providing scientific-based evidence for the clinical use of OMT. By strengthening our understanding of OMT mechanism(s), osteopathic physicians can utilize this

information to treat patients suffering from disease which may reduce morbidity, mortality, and hospitalization.

O_2 (l/min)	1.5	1.5	1.5	1.5	1.5
Temp (F)	101	102	102	102	102
Pulse (BPM)	92	85	88	88	81
Tidal Vol (mL)	540	600	600	600	300
SPO_2 (%)	90	96	99	99	73

Table 1: Pig #1 was placed under anesthesia and after placing the pig in a lateral recumbency position, vital signs were recorded every 30 minutes for the remainder of the surgery (two hours). All vital signs remained in the predicted ranges for anesthetized swine.

O_2(l/min)	1.5	1.5	1.5	1.5	1.5
Iso (%)	3.5	3.5	3.5	3.5	3.5
Temp (F)	101	102	102	101	101
Pulse (BPM)	101	90	75	77	82
Tidal Volume (mL)		700	700	700	700
Condition			Baseline	LPT	Post-LPT

Table 2: Pig #2 was placed under anesthesia and after placing the pig in a lateral recumbency position, vital signs were recorded every 30 minutes for the remainder of the surgery (three hours). All vital signs remained within the predicted ranges for anesthetized swine.

O2 (l/min)	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5	1.5
Temp (F)	100	99	98.7	98.7	98.5	98.4	98.3	98.3	98.3
Pulse (BPM)	124	98	106	102	90	86	86	85	85
Tidal Volume (mL)	600	600	600	600	600	600	600	600	600
SP02 (%)	96	82	94	92	93	98	98	98	98
Condition							Baseline	LPT	Post-LPT

Table 3: Pig #3 was placed under anesthesia and after placing the pig in a lateral recumbency position, vital signs were recorded every 30 minutes for the remainder of the surgery (four hours). All vital signs remained within the predicted ranges for anesthetized swine.

Percentage	Volume of Antibody used	
	10 ul	20 ul
B cells subset	11.6%	10.7%
T cells	14.1%	13.6%
Monulocytes/Granulocytes	19 %	19.1%

Table 4: Two Thoracic duct lymph nodes were collected, homogenized, and combined from pig #3. The leukocytes were stained using two volumes of antibodies, 10 ul (recommended by the manufacturer) and 20 ul. Data are the percentages of the leukocyte subsets.

O2 (l/min)	2	2	2	2	2	2	2	2	2
Temp (F)	99	99.3	99.9	100	100	100.5	100.8	100.8	100.9
Pulse (BPM)	111	87	66	71	71	67	66	64	65
Tidal Volume (mL)	700	700	700	700	700	700	700	700	700
SP02 (%)	90	90	95	93	94	94	93	97	97
Condition			Baseline1	LPT1			Baseline2	LPT2	Post-LPT2

Table 5: Pig #4 was placed under anesthesia and after placing the pig in a lateral recumbency position, vital signs were recorded every 30 minutes for the remainder of the surgery (2.5 hours). All vital signs remained within the predicted ranges for anesthetized swine.

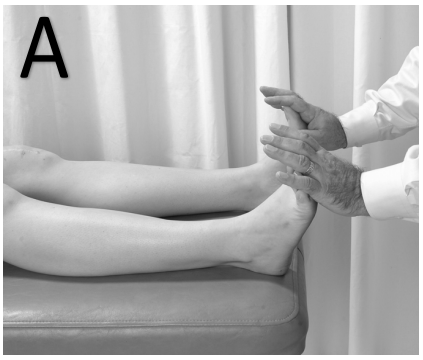

Figure 1A: Pedal pump technique

Figure 1B: Thoracic pump technique

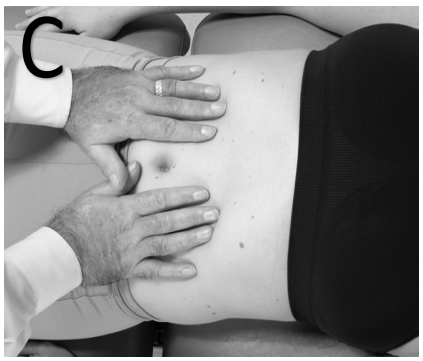

Figure 1C: Abdominal pump technique

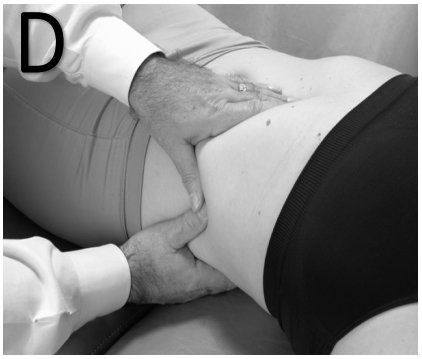

Figure 1D: Splenic pump technique

Figure 1: Images taken from Hruby & Hoffman, 2007. OMT techniques most commonly used to increase lymphatic flow in clinical disease. Figure 1A is represents the pedal pump where the physician repeatedly pushes upward on the feet of the patient to create an oscillatory force to mobilize fluid in the lower extremity and the whole body. Figure 1B is a representation of the thoracic pump being applied. Upon exhalation the physician will push downward on the thoracic cage by applying posterior and inferior compression. Figure 1C represents the abdominal pump technique. The physician places his/her hands below the costo-diaphragmatic junction and pressures are exerted medially and cranially to compress the abdomen repeatedly to increase lymph flow. Figure 4D represents the hand placement for the splenic pump technique. During exhalation, the osteopathic physician applies a vibratory motion with both hands to encourage lymphatic pumping of the spleen.

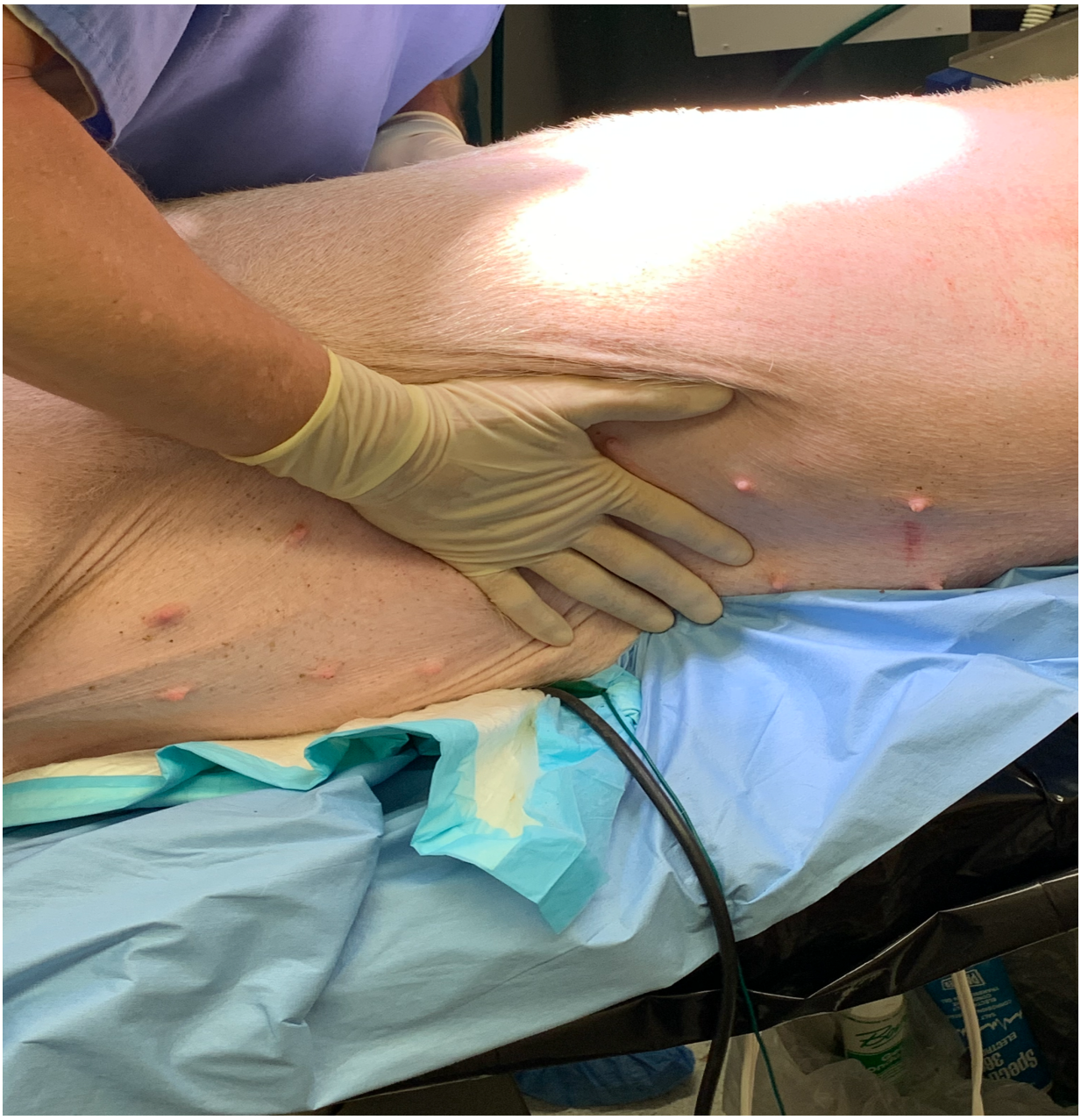

Figure 2: Pig #4 was placed in a supine position. A member of our surgical team, Dr. Tune, contacted the ventral side of the pig's abdomen with his hands placed bilaterally below the cost-diaphragmatic junction. Pressure was exerted medially and cranially to compress the abdomen until resistance was encountered against the diaphragm, and then the pressure was released. Abdominal compressions were administered approximately once per second for a total of four minutes.

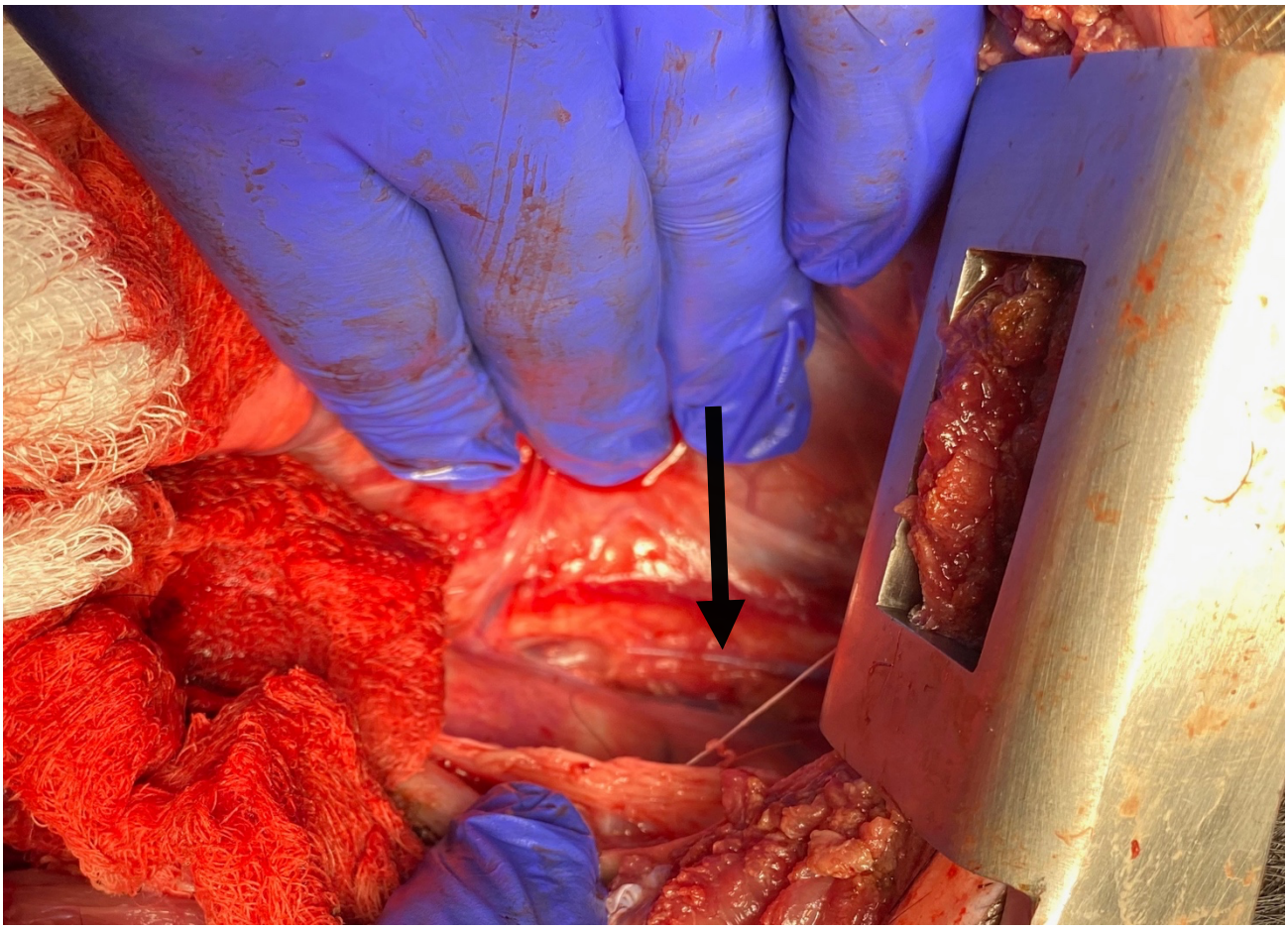

Figure 3: Pig #1 was placed under anesthesia and then placed in a lateral recumbency position. The surgical team gained access to the thoracic duct via thoracotomy. The thoracic duct runs along the descending aorta in pigs.

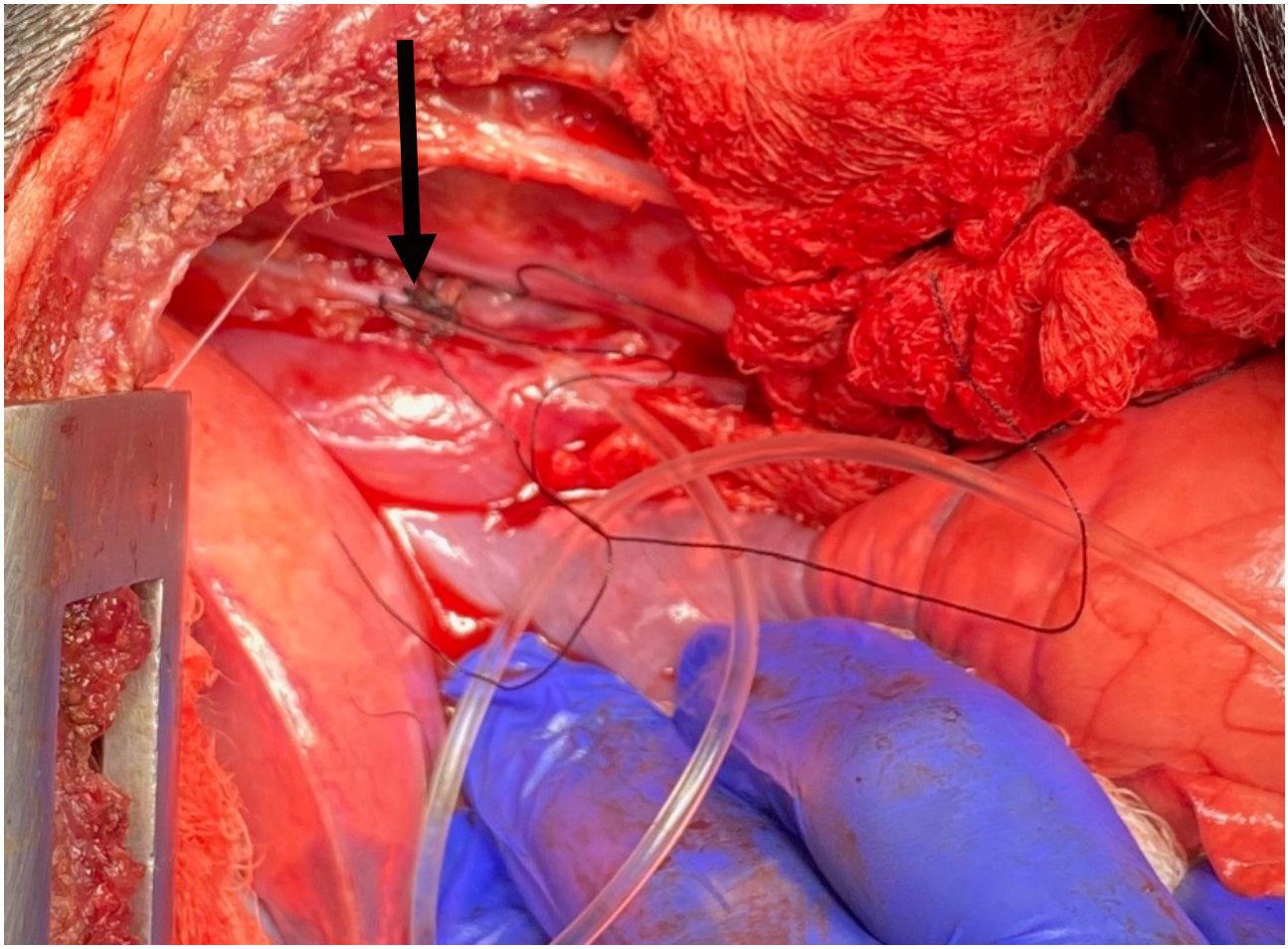

Figure 4: Pig #1 was anesthetized and placed in a lateral recumbency position. The thoracic duct was accessed via thoracotomy. An 18' angio-catheter was placed inside the thoracic duct to collect TDL lymph during baseline, LPT, and recovery periods.

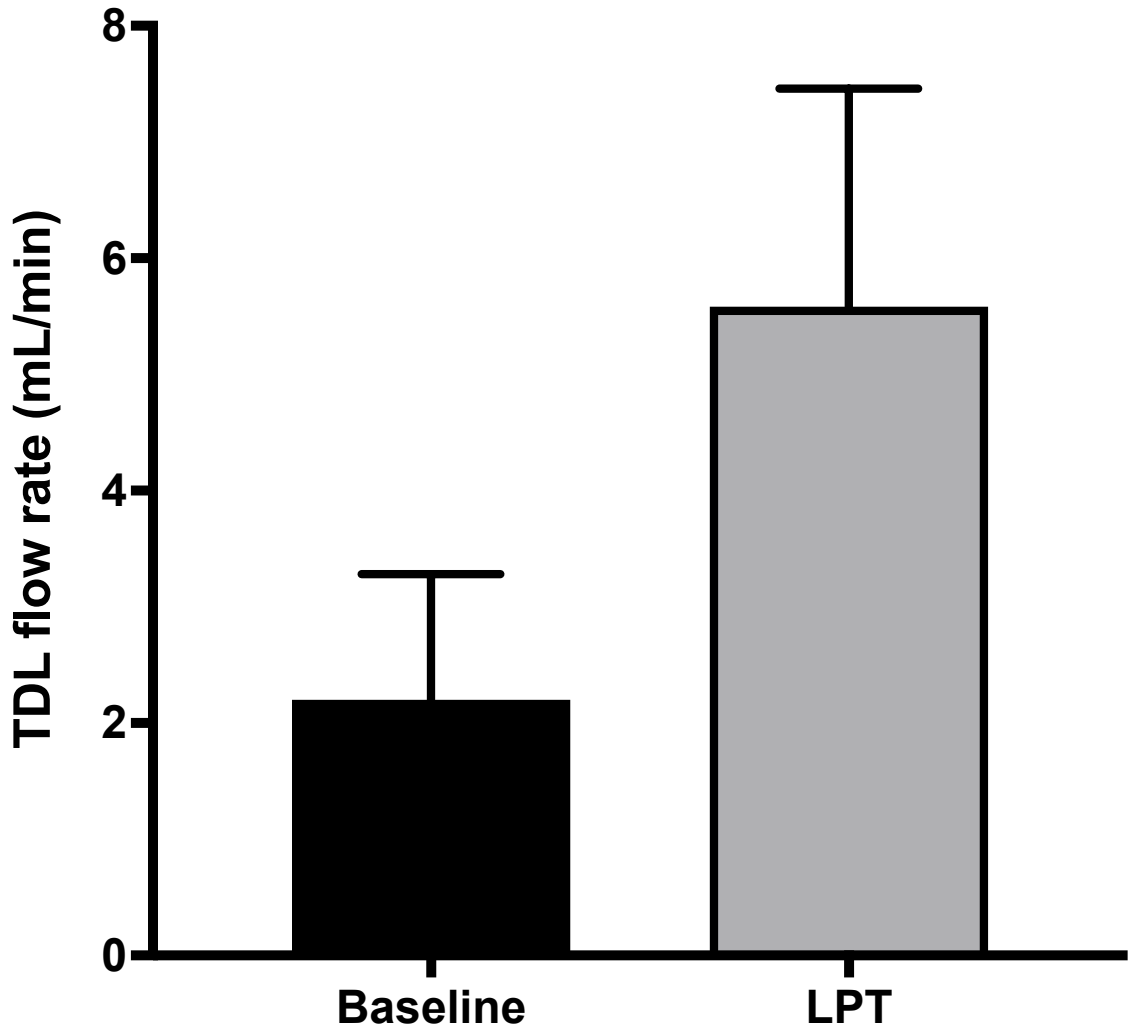

Figure 5: The thoracic duct of Pigs #1 and #3 were exposed then cannulated. Thoracic duct lymph (TDL) was collected during three conditions: baseline, LPT, and recovery. TDL flow rate was calculated during baseline and LPT conditions by timed collection. Data are reported as mean ± STD. The average baseline flow rate was 2.2 $\pm$ 1.0 mL/min, and the average LPT flow rate was 5.58 $\pm$ 1.8 mL/min.

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