In Vivo Investigations into the Effects of Pathological Conditions Including Diabetes on Lymphatic Function and Wound Healing

Yusupjan Abdulla
M.Med

A thesis submitted in fulfilment of the requirements for the degree of Doctor of Philosophy
February 2016

School of Biomedical Sciences and Pharmacy
University of Newcastle
Statement of Originality

This thesis contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text. I give consent to this copy of my thesis, when deposited in the University Library, being made available for loan and photocopying subject to the provisions of the Copyright Act 1968.

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Statement of Authorship

I hereby certify that the work embodied in this thesis contains a published paper/s/scholarly work of which I am a joint author. I have included as part of the thesis a written statement, endorsed by my supervisor, attesting to my contribution to the joint publication/s/scholarly work.

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University of Newcastle
Dedication

To my beloved father Abdulla Matsiyit who was my first teacher in science, and my dear mother Ruzinisahan Yusuf who has given her endless love and always believed in me and my success. I always love them forever and pray for them.
Acknowledgement

I would like to express my sincere gratitude to my supervisors Prof. Dirk van Helden, Dr. Mohammad Imtiaz, Dr. Phillip Jobling and A/Prof. Derek Laver for the continuous support of my PhD study and research, for their patience, motivation, enthusiasm, and immense knowledge. I thank Peter Dosen for his excellent technical assistance and sharing his life experience. I express my appreciation to Gough Au for kind assistance in measuring cytokines. I thank all the people around me for their support. I thank my parents for their education, which has been great impact on my life. I also thank all my brothers and sister who have supported me in many ways. I sincerely thank my wife Adalat Jurat and my lovely children for being with me and supporting me for this period of time.
### Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
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<tr>
<td>ABP</td>
<td>arterial blood pressure</td>
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<tr>
<td>ACE</td>
<td>angiotensin converting enzyme</td>
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<td>AIHW</td>
<td>Australian Institute of Health and Welfare</td>
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<td>ARBs</td>
<td>angiotensin receptor blockers</td>
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<td>BSA</td>
<td>bovine serum albumin</td>
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<td>(\text{Ca}^{2+})</td>
<td>calcium ion</td>
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<td>CCBs</td>
<td>calcium channel blockers</td>
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<td>CCD</td>
<td>charge-coupled device</td>
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<td>Cl(^-)</td>
<td>chloride anion</td>
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<td>Cl(_{\text{Ca}})</td>
<td>Ca(^{2+})-activated Cl(^-) channels</td>
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<td>cm</td>
<td>centimeter</td>
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<td>DM</td>
<td>diabetes mellitus</td>
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<td>DMSO</td>
<td>dimethyl sulfoxide</td>
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<td>EDD</td>
<td>end-diastolic diameter</td>
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<td>EF</td>
<td>ejection fraction</td>
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<tr>
<td>EMCCD</td>
<td>electron multiplying charge-coupled device</td>
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<tr>
<td>ESD</td>
<td>end-systolic diameter</td>
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<tr>
<td>FKBP12</td>
<td>12-kDa tacrolimus binding protein</td>
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<td>FREQ</td>
<td>contraction frequency</td>
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<td>g</td>
<td>gram</td>
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<tr>
<td>GRO/KC</td>
<td>growth-regulated oncogene/ keratinocyte chemoattractant</td>
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<td>H</td>
<td>hypothesis</td>
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<td>HR</td>
<td>heart rate</td>
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<td>i.d.</td>
<td>intradermal</td>
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<td>I.D</td>
<td>inside diameter</td>
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<td>i.p.</td>
<td>intraperitoneal</td>
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<td>i.v.</td>
<td>intravenous</td>
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<td>ICCs</td>
<td>interstitial cells of Cajal</td>
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<td>ICG</td>
<td>indocyanine green</td>
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<td>If</td>
<td>funny current</td>
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<td>IL-6</td>
<td>interleukin-6</td>
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<tr>
<td>IP(_3)R</td>
<td>inositol 1,4,5-trisphosphate receptor</td>
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<tr>
<td>K(^+)</td>
<td>potassium ion</td>
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<tr>
<td>K(_{\text{ATP}})</td>
<td>ATP-dependent potassium channels</td>
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<td>kg</td>
<td>kilogram</td>
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<tr>
<td>L</td>
<td>length</td>
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<tr>
<td>LEC</td>
<td>lymphatic endothelial cell</td>
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<tr>
<td>LPS</td>
<td>lipopolysaccharides</td>
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<td>MAP</td>
<td>mean arterial blood pressure</td>
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<td>MCP-1</td>
<td>monocyte chemoattractant protein-1</td>
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<td>mg</td>
<td>milligram</td>
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<td>ml</td>
<td>milliliter</td>
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<td>Abbreviation</td>
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<td>MLV</td>
<td>mesenteric lymphatic vessels</td>
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<td>mmHg</td>
<td>millimeter of mercury</td>
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<td>mmol</td>
<td>millimolar</td>
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<tr>
<td>Na⁺</td>
<td>sodium ion</td>
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<tr>
<td>nAMP</td>
<td>normalized contraction amplitude</td>
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<td>NIR</td>
<td>near infrared</td>
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<td>NO</td>
<td>nitric oxide</td>
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<td>O.D</td>
<td>outside diameter</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PO₂</td>
<td>oxygen partial pressure</td>
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<td>ROI</td>
<td>region of interest</td>
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<td>s</td>
<td>second</td>
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<tr>
<td>SAN</td>
<td>sinoatrial node</td>
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<tr>
<td>SERCA</td>
<td>sarco/endoplasmic reticulum Ca²⁺-ATPase</td>
</tr>
<tr>
<td>SR</td>
<td>sarcoplasmic reticulum</td>
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<tr>
<td>STDs</td>
<td>spontaneous transient depolarizations</td>
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<tr>
<td>STZ</td>
<td>streptozotocin</td>
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<tr>
<td>SUR</td>
<td>sulfonylurea receptor</td>
</tr>
<tr>
<td>t</td>
<td>time</td>
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<tr>
<td>T1DM</td>
<td>type 1 diabetes mellitus</td>
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<tr>
<td>TNF-α</td>
<td>tumor necrosis factor-α</td>
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<tr>
<td>V</td>
<td>velocity</td>
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<tr>
<td>VEGF</td>
<td>vascular endothelial growth factor</td>
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<tr>
<td>VIP</td>
<td>vasoactive intestinal peptide</td>
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<tr>
<td>µg</td>
<td>microgram</td>
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<td>µl</td>
<td>microlitre</td>
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<td>µm</td>
<td>micrometer</td>
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Achievements

1. Exchange student grant $3700
2. Three week visit to Harvard University (US) as Visiting Researcher

Submitted articles

Investigating the Effects of Pro-Inflammatory Cytokines Induced by Moderate LPS Administration on Lymphatic Function (Nutrition & Diabetes).

Articles in preparation

1. Lymphatic FunctionRemains Robust in an Acute Diabetic Rat Model
2. Consequences of Glibenclamide on Wound Healing in an Acute Diabetic Rat Model.
3. In Vivo Investigation into the Effects of Nifedipine on Rat Lymphatic Function.

Declaration

These papers constitute the Results Chapters and as such there is some repetition with the Methods. I designed and performed all the experiments plus wrote the first draft for these four papers (i.e. all Results chapters). As confirmed below:

Print name: Dirk F. van Helden

Signature:

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University of Newcastle
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Abstract

Diabetes is a fast growing chronic disease all over the world including Australia. The 1999–2000 Australian Diabetic study indicated that 880,000 Australians had diabetes with most exhibiting type 2 diabetes (96%), and indicated this number increased by 0.7%/year in a 2011-2012 follow-up study. Foot ulcer(s), generally referred to as diabetic foot, is one of the most severe diabetic complications, which combined with infection may lead to foot amputation.

Lymphatic function was investigated in several pathological conditions (i.e. type 1 diabetic mellitus, inflammation and L-type Ca$^{2+}$ channel blocker-treated oedema). Wound healing was also studied, as it has a close link to diabetes and possibly to lymphatic function.

Short- and long-term hyperglycaemia in diabetes has multiple effects. The consequences of hyperglycaemia on the lymphatic system, including the propulsion of lymph, has not been well-studied. We established hyperglycaemia in rats by injecting streptozotocin (STZ), which caused rats to rapidly become hyperglycaemic (from 7 to 18 mmol/l in 1 day) and lose weight (9% reduction 3 days later). We tested lymphatic function 3-5 days after STZ treatment by near infrared (NIR) lymphatic imaging using the dye indocyanine green (ICG) injected into the hind foot of urethane-anaesthetised rats. Lymphatic function measured by contraction frequency and ICG arrival time in exposed groin lymphatic vessels did not differ in control and hyperglycaemic animals. The effect of hyperglycaemia in the initial stage of diabetes on contraction frequency and first arrival time is subtle or compensated such that lymphatic vessels function normally.

Understanding and treating diabetic wounds remains a major challenge. Numerous studies demonstrate that hyperglycaemia hinders wound healing through multiple pathways. It is also known that glibenclamide, an anti-diabetic drug, slows down the healing process in various non-diabetic experimental wound models. The direct effect of hyperglycaemia and glibenclamide on wound healing is poorly understood. Streptozotocin (STZ) -treated rats, which were markedly hyperglycaemic even 1 day after treatment, had a small circular skin wound made on an upper hind foot under
anaesthesia on day 3 after STZ treatment. A corresponding control group had the same skin wound made. Wound healing was compared for 3 conditions (control, STZ treatment; STZ treatment with glibenclamide cream applied to the same hind limb) by taking daily images of the wound with assessment of healing made by measuring parameters such as: the percentage wound area; the average time for the wound to close to a specified percentage; the time to complete wound closure; and the linear advance of the wound edge. We determined that glibenclamide only had a small negative effect on wound healing. This adverse effect is likely to be well compensated by its normal hypoglycaemic role in treatment of type 2 diabetes.

Severe systemic infection and resultant inflammation have been shown to suppress rat lymphatic contractile activity and hence the propulsion of lymph through increased cytokines. In clinical medicine, infection and associated inflammation often occur at low to moderate levels. In order to investigate the effects on lymphatic function of a low to moderate level of infection/inflammation, inflammation was induced by injecting a low dose of lipopolysaccharides (LPS at 1.65 mg/kg) into the rat foot. The function of the inguinal-to-axillary lymphatic vessels was assessed by performing near infrared (NIR) imaging after LPS-induction of inflammation, with the level of inflammation assessed by cytokine measurement from blood. Inflammation was induced, as cytokines interleukin-6 (IL-6), monocyte chemoattractant protein-1 (MCP-1) and the chemokine GRO/KC were all significantly increased. In contrast, spontaneous lymphatic contraction frequency and contraction wave velocity were not altered by low dose LPS injection though overall lymph movement, as assessed by the summed distance the waves of contraction moved in a 5 minute period was increased compared to saline control. Increased lymphatic function assessed with 5-minute lymph travel distance ($L_5$) is possibly consistent with increased oedema caused by the LPS. The $K_{\text{ATP}}$ channel blocker glibenclamide did not alter inflammation, as the cytokine levels and lymphatic function were not significantly changed indicating $K_{\text{ATP}}$ channels were not involved in the regulation of cytokine production.

Peripheral oedema is one of the common complications of L-type Ca$^{2+}$ channel blocker drugs in hypertensive patients. We hypothesized that compromised lymphatic function would hinder fluid transportation from the interstitial space due to reduction in lymphatic contractile activity by calcium channel blockade. We tested whether rat
lymphatic function is compromised during nifedipine inhibition of L-type Ca\(^{2+}\) channels using near infrared (NIR) imaging of groin vessels. Nifedipine solution was infused 200 µg/kg/min or 400 µg/kg/min intravenously in deeply anesthetised rats in which arterial blood pressure was monitored. Contraction frequency and contraction wave velocity were monitored \textit{in vivo} using NIR. Although both 200 µg/kg/min and 400 µg/kg/min nifedipine caused significant reduction in arterial blood pressure, lymphatic vessel contraction frequency and contraction wave velocity did not alter during nifedipine infusion. Lymphatic contraction frequency and contraction wave velocity could be obtained in a quantifiable manner through NIR imaging of the rat groin. Nifedipine, an L-type calcium channel blocker, at doses that caused significant reduction in arterial blood pressure, did not reduce lymphatic drainage. Therefore, other factors such as increased filtration in the microcirculation are likely to be a major cause of nifedipine-related peripheral oedema.

Finally we note that during the above-mentioned experiments we investigated rat lymphatic contractile activities in different regions (i.e. deep groin, groin and inguinal-to-axillary lymphatics) finding the contractile activity differed in these regions. A new finding made during these studies was demonstration of two lymphatic pathways from the tail injection site to the inguinal lymph node.
Chapter 1. General Introduction

1.1 Lymphatic system

1.1.1 Introduction

The lymphatic system forms part of the circulatory system. Lymphatic vessels have been observed as a dense network, paralleling that of blood vessels, throughout most of the body except the brain (Casley-Smith 1983). Although a recent study showed lymphatic vessels extended into the brain (Louveau, Smirnov et al. 2015). The main function of the lymphatic system is to collect excess fluid from the interstitial space and transport it into the circulation to maintain homeostasis (Mayerson 1963). There are also other roles including absorption of lipids from the intestine and transport of immune cells to and from lymph nodes (Casley-Smith 1962, Witte, Way et al. 1997).

Figure 1.1: The lymphatic system: old (left) and new (right) concept

A novel study showed lymphatic vessels and nodes are not only distributed widely over the body also including the brain (Louveau, Smirnov et al. 2015). The figure is modified from (Barney 2015)
1.1.2 Lymph fluid

The composition of lymph fluid is similar to that of plasma and interstitial fluid. The concentration of small molecules and ions in lymph is close to the level in the serum. In contrast, the concentration of proteins in lymph, mainly derived from plasma, are generally lower than that of plasma (Zawieja and Barber 1987). The lymphatics of the gastrointestinal tract and some glands present an exception to this rule (Keiding 1966). In addition to containing ions, small molecules and proteins, lymph also contains lymphocytes, macrophages and other cells of the immune system.

Lymph fluid has a relatively low oxygen partial pressure (PO$_2$) compared with arterial blood being around 25 - 40 mmHg depending on the metabolism and vascular perfusion of the surrounding tissue (Hangai-Hoger, Cabrales et al. 2004, Hangai-Hoger, Tsai et al. 2007).

1.1.3 Lymph nodes

Lymph nodes are kidney-shaped (size 1mm-1cm in diameter) immune organs (Fig. 1.2) connected by collecting lymphatic vessels. Most lymph passes through at least one lymph node before entering the blood stream (Drinker, Field et al. 1934, Engeset 1959) except for some organs (e.g. testicle) (Engeset 1959).

The primary role of lymph nodes is to provide immune surveillance centres to produce immune competent cells (e.g. lymphocytes) leading to antibody secretion (Gretz, Anderson et al. 1997). Particulate matter in lymph can be filtered and destroyed by macrophages.
Figure 1.2: Lymph node: An idealized section of a small lymph node that contains three lymphoid lobules

Each lobule is centred under its own afferent lymphatic vessel. Arterioles (red) and venules (blue) are present in the medullary cords. The lobules exhibit a reticular meshwork superimposed on the vasculature. The centre lobule is separated from the left lobule by a transverse sinus. The right lobule shows a lobule as it appears in histological section. Modified from (Willard-Mack 2006)
1.1.4 Structure and function of lymphatic vessels

There are two types of lymphatic vessels: 1) initial lymphatics, where fluid enters the system and, 2) collecting lymphatics, which transport the lymph to the blood stream.

1.1.4.1 Initial lymphatics

Initial lymphatics are blind-ended tubes that consist of a single layer of flattened, non-fenestrated endothelial cells (Leak 1976, Azzali and Arcari 2000). Alternative names for these include terminal lymphatics, peripheral lymphatics and lymphatic capillaries (Fig. 1.3). The lymphatic endothelium plays an important role in the formation of lymphatic vessels (lymphangiogenesis), metastasis and embryonic lymphatic development. The initial lymphatics collect interstitial tissue fluid, solutes, cells and particulate matter (Mayerson 1963, Leak 1980). In most species they do not have a muscle layer (Leak 1980), but may have actin-like filaments within their endothelial cells (Casley-Smith and Florey 1961, Leak 1970, Castenholz 1987).

The size of the initial lymphatics ranges from 5-60 µm in diameter (Casley-Smith and Vincent 1980, Ushiki 1990). The cytoplasmic extensions of endothelial cells overlap at the cell junction to form pores that allow interstitial fluid to enter the initial lymphatics. This is the primary valve system in the lymphatics (Trzewik, Mallipattu et al. 2001). The outer surfaces of initial lymphatic endothelial cells are connected to the matrix fibre by anchoring filaments of collagen VII (Sakai, Keene et al. 1986).

Figure 1.3: Initial lymphatics

When tissue pressure is increased, the flaps are moved inward and gaps open, and fluid flows into the initial lymphatic vessel. When pressure inside the lymphatic vessel is higher than tissue fluid pressure, the flaps are pressed outward and the gaps are closed. Modified from (Yu, Morgan et al. 2009)
The normal pressure in the interstitial space for forming lymph ranges from -7 mmHg to +2 mmHg. If the interstitial fluid pressure increase beyond this level, fluid may accumulate in the interstitium leading to oedema (Taylor 1990).

There are three types of junctions between endothelial cells in the initial lymphatics, tight junctions, open junctions and close or narrow junctions. The open junctions play the most important role in lymph formation. The number of junctions depends on the region of body and the conditions of surrounding tissue. The number of open junctions can be increased by 20-40% when there is an oedema (Casley-Smith 1983).

1.1.4.2 Collecting lymphatics
As stated above, the main function of the lymphatic system is to collect lymph and transport it to the blood stream (via thorax duct converging at the junction of the internal jugular vein and the subclavian vein). Movement of lymph towards the vena cava is facilitated by extrinsic driving force, such as skeletal locomotor muscles, movements of the gastrointestinal tract and the actions of the respiratory muscles (Leak, Schannahan et al. 1978, Mazzoni, Skalak et al. 1990). In addition to these important drivers, collecting lymphatics have a medial layer of one to three layers of smooth muscle cells. These smooth muscle cells are capable of spontaneous rhythmic contractions (Mislin 1976, Van Helden 1993). In this context, bulk movement of lymph is largely dependent on the unique anatomy of the collecting lymphatics.

Collecting lymphatics consist of serial lymphangions (Fig. 1.4), which are the functional units of lymphatic pumping. Lymphangions are delimited by unidirectional upstream and downstream valves that prevent backflow during the constriction (Mislin 1976).

Collecting lymphatics contain three layers, an intimal monolayer of endothelial cells, a media comprised of one to three layers of smooth muscle cells and an adventitia. (See collecting lymphatic below)
Figure 1.4: Collecting lymphatics

An arrow showing a guinea pig mesenteric lymphatic. The narrow part of the vessel is where the valves are located. Lymphangions are the chambers situated between consecutive valves. Modified from (Van Helden 1993).
**Lymphatic endothelium**

Lymphatic endothelial cells form the inner layer of collecting lymphatics with these cells connected by tight junctions (otherwise referred to as close junctions). A continuous basement membrane closely attached to lymphatic endothelium provides support and further minimizes the permeability of the vessels.

**The media**

The number of the smooth muscle layers within the media increases and becomes more ordered as vessels progress towards the thorax duct. The number of smooth muscle layers is also species dependent (Ohhashi, Fukushima et al. 1977, Von der Weid 2001). In smaller animals, only one smooth muscle layer is present, which is oriented circularly.

The smooth muscle in collecting lymphatics within some skeletal muscles are absent as skeletal muscle contraction is sufficient to propel the lymph (Aukland and Reed 1993). The smooth muscle in other collecting lymphatics constricts by spontaneous pacing mechanisms to propel lymph (Kirkpatrick and McHale 1977, Van Helden 1993).

**The adventitia**

The outer layer of collecting lymphatics is termed adventitia or tunica adventitia and contains fibroblast cells, connective tissue, blood vessels and nerves (Yoffey and Courtice 1970). The collecting lymphatics do not contain filaments to connect to surrounding tissue (Casley-Smith 1969).
Figure 1.5: This scanning electron micrograph illustrates the appearance of a valve as seen in a cross section of a lymphatic vessel.

A pair of leaflets extend from the wall in a circumferential fashion and project into the lumen of the vessel at such an angle that their free edges fit tightly together (x 240). Modified from (Leak 1980)
1.1.5 Innervation

The presence of nerves associated with lymphatic vessels was observed in various species in the last century. Sympathetic, parasympathetic and sensory nerves have all been shown to innervate lymphatic vessels. Immunohistochemical studies made on mesenteric lymph vessels in the guinea pig revealed both cholinergic and adrenergic nerve fibers (Alessandrini, Gerli et al. 1981). Evidence for a functional role of nerves is provided by various experiments including the demonstration that frequency-related contractions produced by electrical stimulation in isolated dog thoracic duct were abolished in the presence of nerve block by tetrodotoxin and inhibited by antagonists of alpha1- and alpha2-adrenoceptors (Igarashi, Ikomi et al. 1998). This indicates that adrenergic excitatory nerve fibers innervate the wall of dog thoracic ducts. It has been demonstrated by immunohistochemistry that VIP (Vasoactive Intestinal Peptide) containing nerve fibers innervate bovine mesenteric lymphatic vessels (Ohhashi, Olschowka et al. 1983). These are likely to cause vessel relaxation, as direct application of VIP was shown to induce a concentration-dependent relaxation of isolated bovine mesenteric lymphatics (Ohhashi, Olschowka et al. 1983).

Innervation of lymphatic vessels is therefore likely to strongly contribute to lymphatic function by modulating the frequency and amplitude of contractions and hence controlling lymph flow (McHale, Roddie et al. 1980). This could be of great significance in diabetes, as neuropathy is one of the complications of diabetes. Thus impaired innervation of lymphatics also may reduce lymphatic pumping in diabetic patients.

1.1.6 Lymphatic smooth muscle

Lymphatic smooth muscle is similar to vascular smooth muscle consisting of elongated, fusiform cells with tapering ends, 150-200 µm in length and 3-6 µm in diameter. The cell membrane contains various functional proteins such as receptors, ion channels, pumps, ion exchangers and gap junctions. Gap junctions connect the cytoplasm of adjacent cells, which allow ions and molecules to pass through freely carrying electrical and metabolic signals between neighbouring cells. In addition to membrane receptors and channels there are many intracellular structures such as the sarcoplasmic reticulum (SR), mitochondria, dense bodies and caveolae, which are also critical to cell functions including muscle contraction (Fig 1.6).
Figure 1.6: Schematic illustration of the proposed hypothesis for lymphatic smooth muscle contraction modified from (von der Weid and Zawieja 2004)

1.1.6.1 sarcoplasmic reticulum
The sarcoplasmic reticulum (SR) of smooth muscles consists of the peripheral SR (~15-30nm from the plasma membrane) and the central SR located further within the cytoplasm (Poburko, Kuo et al. 2004, Sweeney, Jones et al. 2006). Peripheral SR is associated with electrical excitability whereas central SR is directly involved in muscle contraction (Wray and Burdyga 2010). It should be noted that most of our knowledge of excitation contraction coupling in smooth muscles is derived from tissues other than lymphatics, e.g. arterial and gastrointestinal tract muscle. The volume of the SR in rabbit pulmonary artery and aorta account for ~5% of the cell volume and this value is decreased to ~2% in mesenteric vein, artery and portal vein. (Devine, Somlyo et al. 1972).

Accumulated studies have shown that SR supplies much of the intracellular calcium for smooth muscle contraction (Endo, Yagi et al. 1982, Bond, Kitazawa et al. 1984, Somlyo, Walker et al. 1988). Following calcium release and contraction, the SR is refilled by the SERCA-pump (sarco/endoplasmic reticulum Ca$^{2+}$-ATPase). This release and reuptake of calcium is important for rhythmic contractions (Goldbeter, Dupont et al. 1990, Meyer and Stryer 1991).
1.1.6.2 Inositol 1,4,5-trisphosphate receptor (IP$_3$R)

IP$_3$ receptors (IP$_3$Rs) are calcium ion (Ca$^{2+}$) release channels located on the SR membrane. IP$_3$Rs are regulated by IP$_3$, Ca$^{2+}$ and modulated by adenine nucleotides, pH, phosphorylation, redox state and various accessory proteins including ankyrin, calmodulin, FKBP12 (12-kDa tacrolimus binding protein), and possibly others (Taylor 1998). A single IP$_3$R is composed of four subunits, and each subunit consists of a NH$_2$ terminus, a hydrophobic region comprising 6 transmembrane domains, and a COOH-terminal tail (Fig 1.7) (Michikawa, Hamanaka et al. 1994, Yoshikawa, Morita et al. 1996).

There are three different types of IP$_3$Rs, each encoded by different genes (Patel, Joseph et al. 1999, Iino 2000, Foskett, White et al. 2007). IP$_3$R1 is mainly expressed in the central nervous system (Furuichi, Yoshikawa et al. 1989). IP$_3$R2 is distributed predominantly in hepatocytes and cardiac myocytes (Iino 2000). In smooth muscle, all three types of IP$_3$Rs are present, but the relative level of each isoform varies in different smooth muscles (Narayanan, Adebiyi et al. 2012). The dominant type of IP$_3$R isoform in lymphatic smooth muscle remains unknown.
Figure 1.7: Inositol 1,4,5-trisphosphate receptor (IP₃R) molecular structure.
A: Schematic representation of an IP₃R subunit depicting important domains and regions. Sites for ATP-binding (black squares), Ca²⁺-binding (blue squares), and phosphorylation (red squares) are indicated. B: Single IP₃R subunit illustrating important domains, regions, and pore. C: Tetrameric IP₃R channel. SR, sarcoplasmic reticulum; N, NH₂ terminus; C, COOH terminus, TRPC, transient receptor potential cation. Modified from (Narayanan, Adebiyi et al. 2012).
When Ca\textsuperscript{2+} changes have been visualized in smooth muscle using fluorescent probes, a variety of spontaneous increases in Ca\textsuperscript{2+} have been observed, each with a characteristic time course and magnitude. These have been termed Ca\textsuperscript{2+} flashes, oscillations, ripples, puffs, sparks, sparklets and waves (Miriel, Mauban et al. 1999, Boittin, Coussin et al. 2000, Jaggar, Porter et al. 2000, Navedo, Amberg et al. 2005, Balemba, Salter et al. 2006, Zhao, Adebiyi et al. 2008). These Ca\textsuperscript{2+} signals play important roles in physiological functions, including contractility, gene expression, migration, and proliferation.

1.1.7 Lymphatic pumping

Extrinsic and intrinsic pumping forces carry out lymphatic pumping function. Intrinsic lymphatic pumping is mediated by a rhythmic contraction-relaxation cycle of the smooth muscle (McHale and Roddie 1975). Pacemaker activity generates an action potential and resultant contraction in lymphatic smooth muscle cells, however a specific pacemaker cell type has not been identified. McCloskey reported putative pacemaker cells in sheep mesenteric lymphatic vessels that may contribute to the pace-making activity (McCloskey, Hollywood et al. 2002). These cells were identified by immunohistochemistry as being positive to the protein c-Kit, which is a marker for pacemaker cells in the gastrointestinal tract known as interstitial cells of Cajal (ICCs). Pacemaker-induced triggering of an action potential brings in Ca\textsuperscript{2+} through L-type channels, which then causes contraction. The contraction of lymphangions then acts as a primitive heart to propel lymph forward through the unidirectional valves.

1.1.7.1 Measurement of lymphatic pumping function

There are several parameters used to evaluate lymphatic vessel function (Zawieja, Wang et al. 2012). These include:

1) End-diastolic diameter (EDD) - the diameter of a vessel when fully dilated;
2) End-systolic diameter (ESD) - the diameter of a vessel when fully constricted;
3) Normalized contraction amplitude (nAMP) - the value between the end-diastolic diameter (EDD) and end-systolic diameter (ESD) normalized to the end-diastolic diameter (i.e. \( nAMP = \frac{EDD - ESD}{EDD} \));
4) Contraction Frequency (FREQ) - the number of contractions per minute;
5) Ejection fraction (EF) – the volumetric fraction of lymph pumped with each
contraction ($EF = (EDD^2 - ESD^2)/EDD^2$)); and
6) Fractional pump flow (FPF) - an index of lymph outflow per minute ($FPF = EF \times FREQ$).
7) Amplitude-Frequency Product (AFP) - an index of lymph pump flow ($AFP = nAMP \times FREQ$)

Figure 1.8: Representative diameter tracings of mesenteric lymphatic vessels (MLV) in vivo constriction data (solid lines) obtained in a rat mesenteric vessel overlapped with corresponding lymph flow velocity (data points) (Akl, Nagai et al. 2011). End-diastolic diameter (EDD) and the end-systolic diameter (ESD) are the dilated and constricted diameters of the vessel respectively.

In addition to diameter tracking of lymphatic edges, near infrared lymphatic imaging, has been used to provide information on lymphatic function, such as contraction frequency and contraction wave velocity. Another in vivo procedure whereby the diameter change and stroke volume as well as the above parameters can be obtained in vivo is through a lymphatic imaging procedure, in which lymphatic vessels are visualised with FITC-dextran stimulated by visible light (Liao, Cheng et al. 2011, Liao, Jones et al. 2014).

1.1.8 Pacemaking mechanisms in lymphatic smooth muscle
Two mechanisms for pacemaking have been proposed for lymphatic smooth muscle these being a $Ca^{2+}$ store-controlled pacemaker model and a classical (cardiac-like)
membrane pacemaker model. Present research indicates that both pacemaker mechanisms subserve a role with the two combining to pace lymphatic vessels (van Helden 2014, van Helden, von der Weid et al. forthcoming 2016).

1.1.8.1 Ca\(^{2+}\) store-controlled pacemaker

In this model of lymphatic pacemaking, rhythmical Ca\(^{2+}\) release from intracellular Ca\(^{2+}\) stores activates Cl\(^-\) channels in the cell membrane to produce spontaneous transient depolarizations (STDs) for each release event. These summate to produce pacemaker potentials that trigger an action potential and muscle constriction (Van Helden 1993). Here rhythmicity is considered to be determined by the store release-refill cycle with stores summating their activity by interacting as coupled oscillators (Van Helden and Zhao 2000, van Helden and Imtiaz 2003).

1.1.8.2 cardiac-like model

This is based on the model for heart pacemaking where a hyperpolarization activated inward current, known as \(I_{\text{funny}}\) \((I_I)\), depolarizes the pacemaker cell to reach threshold and trigger an action potential with the cycle then repeating (DiFrancesco 1991). An \(I_I\)-like current has been documented in sheep mesenteric lymphatic smooth muscle, and has been proposed to contribute to pacemaking (McCloskey, Toland et al. 1999). However, as this mechanism is dominant under conditions when vessels are filled (Gui, Scallan et al. 2015, van Helden, von der Weid et al. forthcoming 2016) then distension induced currents presumably arising through activation of stretch activated channels are likely to subserve a major role in pacemaking (van Helden, von der Weid & Imtiaz, 2015).

1.1.8.3 Ionic channels underlying lymphatic pacemaking and the action potential

**Ca\(^{2+}\)-activated Cl\(^-\) channels (Cl\(_{Ca}\))**

Cl\(_{Ca}\) had been reported present in blood vessels and lymphatic vessels (Wang, Hogg et al. 1992, Van Helden 1993). Toland et al. (Toland, McCloskey et al. 2000) studied Ca\(^{2+}\)-activated Cl\(^-\) current in sheep mesenteric lymphatic smooth muscle cells by the perforated patch-clamp technique. Their experiment also indicated that Cl\(^-\) current contributes to both the generation of spontaneous electrical activity with the addition that it also contributed to the plateau phase of the action potential in lymphatic smooth muscle.
The “funny” heart pacemaker channel \( I_f \)

This channel is located on the membrane of pacemaker cells in the heart. The current generated through this channel is called the funny current (\( I_f \)) and is activated by hyperpolarization on cessation of the action potential (DiFrancesco 1981). Subsequent studies have shown that this channel is the hyperpolarization-activated cyclic nucleotide-gated (\( HCN_d \)) channel (DiFrancesco and Borer 2007). Once activated \( I_f \) allows the influx of \( \text{Na}^+ \) ions and efflux of \( \text{K}^+ \) ions, but at these negative potentials the influx of \( \text{Na}^+ \) ions dominates so that \( I_f \) depolarizes the membrane (DiFrancesco 1981).

\( \text{Ca}^{2+} \) channels

These channels are selectively permeable to \( \text{Ca}^{2+} \). One class is voltage dependent activating when the membrane potential is depolarized to specific threshold levels. These are termed voltage-dependent calcium channels (VDCC). The voltage-dependent calcium channels most relevant to lymphatic smooth muscle are T-type \( \text{Ca}^{2+} \) (transient) and L-type \( \text{Ca}^{2+} \) (long lasting) channels. Another class termed ligand-gated calcium channels is activated through binding to a chemical messenger. Examples include the SR \( \text{Ca}^{2+} \) release channels namely the inositol 1,4,5-trisphosphate receptor (\( \text{IP}_3 \)R) and the ryanodine receptor (RyR).

\( T \)-type \( \text{Ca}^{2+} \) channel (\( I_{\text{Ca-T}} \)):

Hagiwara reported the T-type \( \text{Ca}^{2+} \) current (\( I_{\text{Ca-T}} \)) in rabbit sinoatrial node (SAN) pacemaker cells (Hagiwara, Irisawa et al. 1988). Their study showed \( I_{\text{Ca-T}} \) functions as a pacemaker current. This channel may also play an important role in pacemaker activity in lymphatic smooth muscle (Toland, McCloskey et al. 2000).

\( L \)-type \( \text{Ca}^{2+} \) channel (\( I_{\text{Ca-L}} \))

This type of voltage dependent \( \text{Ca}^{2+} \) channel is present in many excitable cells and where present contributes strongly to membrane depolarization and \( \text{Ca}^{2+} \) influx during the action potential. \( I_{\text{Ca-L}} \) belong to the high voltage-activated group of \( \text{Ca}^{2+} \) channels (Moosmang, Lenhardt et al. 2005). There are 4 subtypes of this channel, named \( \text{Ca}_{1.1} \) to \( \text{Ca}_{1.4} \). \( I_{\text{Ca-L}} \) underlies the action potential in lymphatic smooth muscle (Ohhashi, Azuma et al. 1978, Hollywood, Cotton et al. 1997). This current is critical for lymphatic pumping as it generates the action potential. The resultant \( \text{Ca}^{2+} \) entry causes smooth
muscle contraction and blockade of these channels with application of blockers of $I_{\text{Ca-L}}$ (e.g. nifedipine) abolishes pumping (Ohhashi, Azuma et al. 1978, Imtiaz, Zhao et al. 2007).

L-type ($\text{Ca}_{\text{v}1}$) $\text{Ca}^{2+}$ channel blockers (CCBs) are used in clinical practice according to their subtype including dihydropyridine derivatives (i.e. nifedipine and almodipine), phenylalkylamine (i.e. verapamil) and benzothiazepine derivatives (i.e. diltiazem). One of the common complications of nifedipine treatment is local oedema that occurs around the ankle and dorsal aspect of foot, and nearly one-third of nifedipine-treated patients suffer from peripheral oedema, causing some 8% of them to withdraw from treatment. The oedema caused by CCBs is considered to be due to increased permeability of postcapillary venules (Taherzadeh, Das et al. 1998) and increased intra-capillary hydrostatic pressure (Valentin, Ribstein et al. 1989). As described above, the lymphatic system is closely linked to the fluid balance, therefore it is necessary to examine lymphatic pumping with CCB administration in order to investigate whether CCBs contribute to oedema via reduced lymphatic pumping. The in vitro effect of CCBs, such as nifedipine, reduce lymphatic smooth muscle contraction strength at about 300 nM without altering contraction frequency with 500 nM nifedipine completely inhibiting lymphatic contractions (Lee, Roizes et al. 2014). However, this in vitro effect of nifedipine has been not demonstrated in vivo. If lymphatic pumping is decreased, and causes oedema, this makes it a target for treatment of oedema.

**ATP-sensitive $K^+$-channels**

ATP sensitive $K^+$ channels ($K_{\text{ATP}}$ channels) were first discovered in cardiac muscle cells (Noma 1983). PY von der Weid demonstrated the existence of $K_{\text{ATP}}$ channels in guinea pig mesenteric lymphatics, a channel that contributes to the hyperpolarization induced by nitric oxide (von der Weid 1998). They consist of Kir6.x-type and sulfonylurea receptor (SUR) subunits. The SUB2B isoform is present in smooth muscle (Isomoto, Kondo et al. 1996, Yamada, Isomoto et al. 1997). $K_{\text{ATP}}$ channels are have been reported within the plasma membrane, mitochondria and nucleus and have been shown to be located in the plasma membrane (Quayle, Nelson et al. 1997) and mitochondrial membrane (Li, Mizuno et al. 2008) of smooth muscle cells. They are activated by the $K_{\text{ATP}}$ activator diazoxide in mouse pancreatic beta-cells while inhibited by $K_{\text{ATP}}$ channel inhibitors such as tolbutamide (Trube, Rorsman et al. 1986).
Glibenclamide is a selective ATP-sensitive $K^+$ channel blocker and causes inhibition of this current that otherwise would hyperpolarize and hence relax vascular smooth muscle (Standen, Quayle et al. 1989). A study (Mizuno, Ono et al. 1999) indicated that glibenclamide reversed the action of the $K_{ATP}$ agonist pinacidil to decrease spontaneous pumping activity in isolated rat mesenteric lymphatics. However glibenclamide itself did not have significant effects on spontaneous lymphatic contractions. This study concluded that $K_{ATP}$ channels are involved in the regulation of lymphatic spontaneous contractions under pathological circumstances, but not normal conditions, modifying pacemaker activity, contractile activity and/or inter-muscular conduction of the action potential.

$K_{ATP}$ channels of guinea pig mesenteric lymphatic smooth muscle are upregulated in the 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced model for ileitis with nitric oxide (NO) decreasing lymphatic contraction frequency by activation of $K_{ATP}$ channels (Mathias and von der Weid 2013). Glibenclamide restored lymphatic pumping in these isolated lymphatic vessels. Inguinal-to-axillary lymphatic function is significantly inhibited in an LPS-induced inflammation mouse model suggesting that the resultant cytokines inhibit lymphatic pumping, however the involvement of $K_{ATP}$ channels is unclear. Further investigation of this is important, if $K_{ATP}$ channels are upregulated then $K_{ATP}$ channel blockers should enhance lymphatic pumping making these potentially useful drugs in treatment of inflammatory diseases.

**Large conductance $Ca^{2+}$-activated $K^+$-channels (BK channels)**

This type of channel is activated by membrane potential change or increase in intracellular $Ca^{2+}$ concentration (Barrett, Magleby et al. 1982, Nelson, Cheng et al. 1995). Both depolarization of the membrane potential and increases in intracellular $Ca^{2+}$ open BK channels to produce an efflux of $K^+$ and resultant hyperpolarization, which closes most voltage-dependent channels. Cotton et al. reported the $Ca^{2+}$-activated $K^+$ current was mainly due to BK channel current in sheep mesenteric lymphatic smooth muscle (Cotton, Hollywood et al. 1997). Their study suggested that the BK channel current could have a role in repolarizing lymphatic action potentials.
1.1.9 Lymphatic function and glucose level
A study (Li, Mizuno et al. 2008) showed that there is a positive correlation between the concentration of glucose and lymphatic pumping frequency (isolated rat thoracic duct), but this reached a plateau at 3 mM glucose. This study also indicated that a selective mitochondrial $K_{ATP}$ channel blocker (5-hydroxydecanoate) significantly decreased the frequency of lymphatic pump activity whereas the $K_{ATP}$ activator (diazoxide) increased the frequency. Cytochalasin B and phlorizin both inhibited rat thoracic duct pump activity at different glucose concentrations (Li, Mizuno et al. 2008) most likely due to inhibition of glucose transport (Jung and Rampal 1977, Taton, Piatkiewicz et al. 2010).

1.2 Pathophysiology of altered lymphatic function
1.2.1 Lymphatic function and age
Akl et al. investigated the relation between mesenteric lymph flow and age (Akl, Nagai et al. 2011). Comparing adult (9-month old) and aged (24-month old) rats they determined that end-diastolic diameters and end-systolic diameters of mesenteric lymphatic vessels were 71% and 79% greater in aged than adult animals indicating that lymphatic vessels are dilated in aged rats. In contrast, lymphatic contraction frequency was lower in aged compared to adult rats.

1.2.2 Metabolic syndrome and lymphatic function
Zawieja and his colleagues (Zawieja, Wang et al. 2012) investigated the relationship between metabolic syndrome (MetSyn) and lymphatic pumping activity in rats by altering mechanical load capabilities and modulating lymphatic muscle function. The mesenteric lymphatic phasic contraction frequency in the MetSyn group was 30-50% lower than for the controls. As a result, there was a significant reduction in both lymphatic pump flow and fractional pump flow at different intramural pressures (1, 3, 5 cm H$_2$O). However phasic contraction amplitude and tonic index values for lymphatic vessels from MetSyn animals did not demonstrate marked differences compared to the control group. At high external Ca$^{2+}$ concentrations, maximal stimulated force production in mesenteric lymphatics was markedly reduced in MetSyn lymphatic vessels to roughly half of the control value, indicating that the Ca$^{2+}$ sensitivity was significantly decreased in MetSyn lymphatic myofilaments.
Table 1.1: Parameters of the contractile activity of the rat mesenteric lymphatic vessels in vivo in adult and aged animals (Tony J. Akl 2011)

<table>
<thead>
<tr>
<th></th>
<th>Adult-control</th>
<th>Adult L-NAME (15min)</th>
<th>Aged-control</th>
<th>Aged L-NAME (15min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>EDD (µm)</td>
<td>51±3</td>
<td>34±4</td>
<td>87±11</td>
<td>72±6</td>
</tr>
<tr>
<td>ESD (µm)</td>
<td>38±2</td>
<td>27±2</td>
<td>68±7</td>
<td>51±6</td>
</tr>
<tr>
<td>FREQ min⁻¹</td>
<td>9.0±0.6</td>
<td>18.7±1.7</td>
<td>2.9±0.6</td>
<td>10.3±3.4</td>
</tr>
<tr>
<td>FPF min⁻¹</td>
<td>3.9±0.5</td>
<td>6.2±0.6</td>
<td>0.9±0.3</td>
<td>5.5±1.0</td>
</tr>
<tr>
<td>AFP min⁻¹</td>
<td>222±32</td>
<td>348±37</td>
<td>53±16</td>
<td>338±130</td>
</tr>
</tbody>
</table>

Values are means ± SE; n =6 (adult) and n=8 (aged), where n represents the number of animals used for these studies. Control conditions and after application of 100 µM No-nitro-L-arginine methyl ester (L-NAME) are shown. (EDD= End-diastolic diameter, ESD=End-systolic diameter, FREQ= Contraction frequency, FPF= Fractional pump flow, AFP= Amplitude-frequency product; table modified from Akl et al., 2011).

1.2.3 Diabetes and Lymphatics

1.2.3.1 Diabetes

There are 4 classifications for diabetes: type 1, type 2, gestational diabetes and "other types" (Shoback 2011). Type 1 diabetes is a form of diabetes mellitus that is characterized by high blood and urine glucose levels due to lack of insulin following damage of insulin producing beta cells in the pancreas through an autoimmune response (Rother 2007). Type 2 diabetes is the most common type of diabetes mellitus, which is characterized by insulin resistance and relative insulin deficiency (Shoback 2011).

The global incidence of diabetes is rapidly growing. The Australian Institute of Health and Welfare 1999–2000 Australian Diabetic study indicated that 880,000 Australians had diabetes with most (96%) exhibiting type 2 diabetes. The follow-up study conducted in 2011-2012 indicated that annual incidence of diabetes was increased by 0.7% (Tanamas 2013). The Disease Expenditure Database indicated that direct health-care expenditure on diabetes in 2004-05 was $989 million (AIHW 2008).

1.2.3.2 Foot ulcer: A severe complication of diabetes

Foot ulcer is the one of the most severe diabetic complications, which combined with
infections may lead to foot amputation. Foot ulcer occurs in 15% of all diabetic patients and underlies approximately 84% of all amputations (Pecoraro, Reiber et al. 1990). Diabetic foot syndrome presents as a combination of several pathologies, which include foot ulceration neuropathy, peripheral vascular disease and osteomyelitis, which often leads to amputation (Jude and Boulton 1999).

1.2.3.3 Wound healing

Wound healing occurs slowly in diabetic patients due to many factors. These include compromised macrophage function (Maruyama, Asai et al. 2007), slow angiogenic response (Galiano, Tepper et al. 2004, Falanga 2005), decreased growth factor production (Galkowska, Wojewodzka et al. 2006, Goren, Muller et al. 2006), collagen accumulation, epidermal barrier function, decreased granulation tissue (Falanga 2005), keratinocyte and fibroblast migration and proliferation and impaired epidermal nerves (Gibran, Jang et al. 2002).

The normal wound healing process includes four overlapping phases which are coagulation, inflammation, migration-proliferation (including matrix deposition), and remodelling (Falanga 2005). Coagulation arises through platelet aggregation at the wound site and release of fibrinogen fragments and other pro-inflammatory mediators to form a fibrin plug. Inflammatory cells, such as neutrophils and monocytes are recruited at the wound site, and initiate the inflammatory phase. These cells phagocytize bacteria and debris and promote further inflammation. As the inflammatory phase declines, the migration-proliferation phase begins. This includes angiogenesis, collagen deposition, epithelialization, formation of granulation tissue and wound contraction. The final remodelling phase involves collagen deposition and apoptosis of redundant cells (Falanga 2005).

1.2.3.4 Macrophages and wound healing

In the first few days after a wound occurs, circulating monocytes are concentrated into the wound side where they differentiate into macrophages (Odland and Ross 1968, Ross and Odland 1968), phagocytose debris, produce vascular endothelial growth factor (VEGF)-A and -C that regulate tissue repair and induce the formation of lymphatic and blood vessels (Polverini, Cotran et al. 1977, Hong, Lange-Asschenfeldt et al. 2004). The role of macrophages is critical to wound healing with depletion of macrophages at the
wound leading to a delay in wound healing (Leibovich and Ross 1975, DiPietro 1995).

Macrophages have *two critical functions* in the wound healing process.

1) **Efferocytosis**: As cells die they produce an apoptotic load in the wound area where dying cells and debris produce toxic and inflammatory signals. The phagocytosis of apoptotic cells and debris is called efferocytosis (Vandivier, Henson et al. 2006, Khanna, Biswas et al. 2010). This process is essential for resolution of inflammation. Macrophages are key to efferocytosis because they phagocytize bacteria, damaged tissue and dead cells.

2) **Lymphangiogenesis and angiogenesis**: Macrophages, in reaction to a hypoxic environment, secrete vascular endothelial growth factor (VEGF)-A and –C that promote growth of new lymphatic and blood vessel in the wound area (Polverini, Cotran et al. 1977, Hong, Lange-Asschenfeldt et al. 2004).

### 1.2.3.5 Lymphatic function and wound healing

Lymphatic vessels are thought to play a major role in wound healing by maintaining normal interstitial homeostasis by removing the protein-rich lymph fluid from the interstitial space. Importantly they also deliver immune cells to initiate the immune response (Witte, Bernas et al. 2001, Oliver and Detmar 2002).

Slow wound healing often involves delayed removal of debris, persistent oedema and inflammatory cells, events which may in part be caused by reduced lymphangiogenesis (Ji 2005). However, while reduced lymphatic development is a contributing factor to decreased lymphatic removal of debris another contributing factor could be impaired lymphatic pumping in existing vessels. Therefore, understanding lymphatic contractile properties in diabetes may provide a mechanism underlying slow wound healing. We predict that lymphatic pumping is decreased over longstanding type 2 diabetes, which will lead to slow wound healing. The possible underlying mechanism could be that prolonged inflammation causes an upregulation of K\textsubscript{ATP} channels, which slows down lymphatic pumping. Therefore downregulation of this channel (i.e. by K\textsubscript{ATP} channel blockers) might increase lymphatic pumping and accelerate wound healing. However before studying lymphatic function in animal models with long-standing type 2
diabetes, which are difficult to produce, it is useful to start with a type 1 diabetic model. In this regard, it has been shown that lymph volume increased in type 1 diabetes (Feingold, Zsigmond et al. 1985, Moriguchi, Sannomiya et al. 2005), however the underlying mechanisms are still unclear.

1.2.4 Inflammation and Lymphatic Vessels

1.2.4.1 Inflammation

Inflammation is a natural biological response of the body to any damage caused by infection, injury, tumour and tissue necrosis (i.e. infarction). It is the interaction between multiple organs and harmful stimuli, such as blood vessels, lymphatic vessels, leukocytes and other injured organs or harmful stimuli (i.e. chemical, physical and biological damage).

1.2.4.2 Lymphatic vessels in inflammation

Lymphatic vessels play an important role during inflammation. The response of blood vessels to acute inflammation manifests as increased blood flow due to vessel dilation, redness, oedema and pain. These inflammatory responses are achieved by activation of endothelial cells by inflammatory mediators (i.e. interleukin (IL)-6, IL-1β, tumour necrosis factor-α (TNF-α), and vascular endothelial growth factor-A (VEGF-A)) (Pober and Sessa 2007). Lymphatic contractile activity is also regulated by these inflammatory mediators and resultant vascular response (i.e. oedema). An increased interstitial pressure of up to +2 mm Hg elevates lymph formation (Casley-Smith 1983). Subsequently the luminal pressure increases augmenting lymphatic stroke volume (Benoit and Zawieja 1992). In addition to this, open junctions in the end lymphatics increase by ~ 40% (Casley-Smith 1983). The enhanced pumping and increased number of open junctions contribute to decrease oedema. However, some cytokines (i.e. TNF-α, IL-6 and IL-1β) inhibit lymphatic contractile activity as shown in the LPS-induced inflammation model (Aldrich and Sevick-Muraca 2013). Furthermore, lymphatic vessels transport extravasated leukocytes and antigen-presenting cells to lymph nodes, this regulating inflammation. Dense lymphangiogenesis is a characteristic feature of lymphatic remodelling in chronic airway disease (Baluk, Tammela et al. 2005, Nihei, Okazaki et al. 2015), skin inflammation (Kunstfeld, Hirakawa et al. 2004) and rheumatoid arthritis (Zhang, Lu et al. 2007), and this is driven by VEGF-C, VEGF-D (produced by macrophage) and TNF-α (Baluk, Tammela et al. 2005). Inhibition of
VEGFR-3 signalling completely suppressed lymphatic growth (Baluk, Tammela et al. 2005). Lymphangiogenesis is a key factor in airway bronchial lymphedema and exaggerated airflow obstruction (Baluk, Tammela et al. 2005). Tumour necrosis factor-beta, (secreted by T-lymphocytes) also plays an important role in maintaining lymphatic function and inflammation-associated lymphangiogenesis, as demonstrated in mice (Mounzer, Svendsen et al. 2010). In chronic inflammation, fluid can be transported into the circulation via newly formed lymphatic vessels while this can be achieved by enhanced pumping and increased number of open junctions in existing lymphatic vessels.

1.2.4.3 Lymphatic functions in acute severe inflammation (sepsis)

Vascular tone is decreased or lost in severe inflammation/sepsis or shock due to pro-inflammatory cytokines (Sawa, Ueki et al. 2008), overproduction of nitric oxide (NO) (Kiemer, Muller et al. 2002, Aldridge, Razzak et al. 2008) and other factors.

Groeneveld’s study showed that lymph flow volume is increased during human septic shock and decreased on recovery from septic shock (Groeneveld, Teule et al. 1987). The increase in lymph flow volume arises because of the high albumin content of lymph due to increased systemic microvascular albumin flux probably due to increased blood vessel permeability (Groeneveld, Teule et al. 1987). This finding has been demonstrated in numerous animal systemic inflammation/septic shock models (Elias, Johnston et al. 1987, Lattuada and Hedenstierna 2006, Semaeva, Tenstad et al. 2010). However, one of the interesting results among them is that lymph formation remained high even with decreased lymphatic contractile activity (Elias, Johnston et al. 1987).

Before sepsis occurs, another function of lymphatic vessels is to maintain the integrity of the tissue/organ. Jang et al. recently reported that when the lacteals (lymphatic capillaries that absorb dietary lipids in the small intestine and lymph node lymphatics) were ablated by administration of diphtheria toxin, which completely destroyed villi integrity, there was massive intestinal inflammation leading to sepsis (Jang, Koh et al. 2013). This study pointed to the important role of lymphatics in protecting against sepsis.
1.3 Aims and Hypotheses

1.3.1 Hypotheses

The hypotheses of this paper were based on initial pilot data obtained before I joined the laboratory. The data suggested that acute STZ-induced diabetes in rats, with measurements made within a few days after establishment of type 1 diabetes, caused substantial impairment of lymphatic function. While this finding was in contradiction with literature studies of STZ rats where thoracic duct output was measured (Feingold, Zsigmond et al. 1985, Moriguchi, Sannomiya et al. 2005) it remained of particular interest because the pilot data was obtained on limb/groin lymphatics and limbs tend to be the site for major diabetes-associated complications such as diabetic foot. In parallel to this, and as noted above, it had recently been found that inflammatory ileitis caused by TNBS impaired lymphatic function through increasing $K_{ATP}$ channel opening (Mathias and von der Weid 2013). Therefore my starting postulate was that inflammation whether caused by diabetes or other means of inducing inflammation (e.g. LPS) would impair limb/groin lymphatic function and that this impairment would be reversed by inhibition of $K_{ATP}$ channels. It was also predicted that poor lymphatic function would impair wound healing, which would be improved by enhancing lymph flow through blocking $K_{ATP}$ channels with glibenclamide.

The fourth study aimed to investigate another pathological condition that could result from impaired lymphatic function. Specifically I investigated the possibility that impaired lymphatic function was causative in the oedema that often results in patients being treated for hypertension with L-type $Ca^{2+}$ channel blockers. This hypothesis was also not upheld but my findings made in rats were corroborated by findings in humans that appeared in parallel to my studies.
The following are the hypotheses that I based my studies on.

**H1:** Acute STZ-induced diabetes impairs rat limb lymph flow.

**H2:** Wound healing in a rat model of acute STZ-induced diabetes can be improved by blocking lymphatic K\(_{\text{ATP}}\) channels.

**H3:** Low to moderate levels of inflammation impair lymphatic function.

**H4:** Calcium channel blockade reduces *in vivo* lymphatic contractile activity.

In order to test the above hypotheses, the following specific aims will be pursued.

1.3.2 Aims

**Aim 1:** To measure *in vivo* limb/groin lymphatic contractile activity in an acute type 1 diabetes rat model using *in vivo* imaging techniques.

**Aim 2:** To measure the effects of K\(_{\text{ATP}}\) channel blockade on hind foot wound healing in an acute type 1 diabetes rat model.

**Aim 3:** To quantify limb/groin lymphatic function during low to moderate levels of inflammation and test the effects of K\(_{\text{ATP}}\) channel blockade.

**Aim 4:** To assess the effects of a L-type calcium channel blocker on lymphatic function *in vivo*. 
Chapter 2. Methods

2.1 Animals and animal models

All animal protocols and procedures were approved by The University of Newcastle Animal Care and Ethics Committee (approval: A-2009-153). Adult male outbred Wistar rats (age 9-13 weeks) were obtained from the Central Animal Service Unit of the University of Newcastle. All animals were housed for at least 7 days with a 12-hour light/dark cycle before use.

2.1.1 Rat type 1 diabetic mellitus (T1DM) model

Protocol: after 7 days acclimatization, rats were fasted for 6-8 hours. After fasting, body weight and baseline glucose levels were measured, the latter by a portable glucose-meter using blood obtained from tail vein blood collection (see Chapter 3 blood collection section) prior to the induction of diabetes. T1DM was induced by a single intraperitoneal injection of streptozotocin (STZ) solution (Sigma Cat# S0130, 65 mg/kg, 10 mg in 1 ml citrate acid buffer, pH 6.0). Diabetes was generally induced within about 24h as evidenced by the onset of hyperglycaemia, with those above 12 mmol/l deemed diabetic. Control animals received citrate buffer only. After STZ treatment, 10% sucrose water was provided from the day of induction in order to prevent hypoglycaemia from transient hyperinsulinemia due to large β cell destruction and changed back to the normal water on day 3. Food and water access were ad libitum and the cage bedding was changed twice daily. Blood glucose, urine ketones and body weight were monitored daily, with all values and observations recorded on the monitoring sheets and scored. Rats with urine ketones and/or body weight loss ≥ 20%, blood glucose > 33 mmol/l and/or severe illness (overall assessment score > 10 and manifested with tip-toe waking, comatose, unresponsive to extraneous activity or stimulation, see table 2.1) were euthanized.
## Table 2.1 Score sheet - Streptozotocin induced diabetes in the rat (Morton 2000)

<table>
<thead>
<tr>
<th>RAT No:</th>
<th>DATE:</th>
<th>ACEC Approval No:</th>
</tr>
</thead>
</table>

<table>
<thead>
<tr>
<th>INITIAL WEIGHT:</th>
<th>WEIGHT when STZ administered:</th>
</tr>
</thead>
<tbody>
<tr>
<td>DATE</td>
<td></td>
</tr>
<tr>
<td>DAY</td>
<td></td>
</tr>
<tr>
<td>TIME</td>
<td></td>
</tr>
</tbody>
</table>

**SCORES based on details below:**

- **Body weight**
- **Physical appearance**
- **Clinical signs**
- **Normal behaviour**
- **Provoked behaviour**

**Total score**

- **Additional signs**
- **Treatment or Action**
- **Signature**

<table>
<thead>
<tr>
<th>VARIABLE</th>
<th>SCORE</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body weight changes</strong></td>
<td></td>
</tr>
<tr>
<td>Normal - body weight maintained or increased.</td>
<td>0</td>
</tr>
<tr>
<td>Before 7 days post STZ: &lt; 10% Weight loss</td>
<td>1</td>
</tr>
<tr>
<td>Before 7 days post STZ: 10 - 15% Weight loss</td>
<td>2</td>
</tr>
<tr>
<td>&gt; 20% Weight loss from starting weight at any time</td>
<td>Euthanase</td>
</tr>
<tr>
<td>&gt; 7 days post STZ – weighing less than starting weight</td>
<td>Euthanase</td>
</tr>
<tr>
<td><strong>Physical appearance</strong></td>
<td></td>
</tr>
<tr>
<td>Normal, coat is smooth, eyes are clear and bright</td>
<td>0</td>
</tr>
<tr>
<td>Lack of grooming</td>
<td>1</td>
</tr>
<tr>
<td>Piloerection, coat appears rough</td>
<td>2</td>
</tr>
<tr>
<td>Fresh ocular or nasal discharges including porphyrin staining. Evidence of diarrhoea.</td>
<td></td>
</tr>
<tr>
<td>Very rough coat</td>
<td>3</td>
</tr>
<tr>
<td>Abnormal posture (eg. hunched)</td>
<td></td>
</tr>
<tr>
<td>Signs of dehydration (sunken eyes, “square tail”)</td>
<td></td>
</tr>
<tr>
<td><strong>Clinical signs</strong></td>
<td></td>
</tr>
<tr>
<td>Normal blood glucose &lt;150 mg/dl (8.3 mmol/l)</td>
<td>0</td>
</tr>
<tr>
<td>Blood glucose 150 - 300mg/dl (8.3 - 16.7 mmol/l). No ketones in urine.</td>
<td>1</td>
</tr>
<tr>
<td>Blood glucose 300 - 600 mg/dl (16.7 - 33 mmol/l). No ketones in urine.</td>
<td>2</td>
</tr>
<tr>
<td>Blood glucose &gt; 600 mg/dl (&gt; 33 mmol/l). Urine positive for ketones</td>
<td>Euthanase</td>
</tr>
<tr>
<td><strong>Normal behaviour</strong> (eg. when observed from distance)</td>
<td></td>
</tr>
<tr>
<td>Normal behaviour pattern</td>
<td>0</td>
</tr>
<tr>
<td>Minor decrease in activity (eg. less inquisitiveness, inactive when hyperactivity would be expected such as nocturnal activity, new environment). Alert.</td>
<td>1</td>
</tr>
<tr>
<td>Isolated and less mobile but alert.</td>
<td>2</td>
</tr>
<tr>
<td>Immobile, isolated</td>
<td>3</td>
</tr>
<tr>
<td><strong>Provoked behaviour</strong> (in response to external stimuli; eg. when handled)</td>
<td></td>
</tr>
<tr>
<td>Normal</td>
<td>0</td>
</tr>
<tr>
<td>Minor depression/exaggeration of responses</td>
<td>1</td>
</tr>
<tr>
<td>Subdued – responds when stimulated (eg. handling)</td>
<td>2</td>
</tr>
<tr>
<td>Comatose, unresponsive to extraneous activity or stimulation</td>
<td>Euthanase</td>
</tr>
<tr>
<td><strong>Score</strong></td>
<td>If you have scored a 3 more than once, score an extra point for each 3.</td>
</tr>
</tbody>
</table>
## Interpretation of total scores from an overall assessment of an experimental animal

<table>
<thead>
<tr>
<th>Total Score</th>
<th>Overall assessment</th>
</tr>
</thead>
<tbody>
<tr>
<td>0 to 4</td>
<td>Normal to acceptable changes.</td>
</tr>
<tr>
<td>5 to 9</td>
<td>Monitor carefully. Increase frequency of monitoring. Consult with CI and veterinarian.</td>
</tr>
<tr>
<td>10 to 14</td>
<td>Ample evidence of suffering. Consult with CI and veterinarian. Consider euthanasia.</td>
</tr>
<tr>
<td>15</td>
<td>There is ample evidence of severe pain. Animal is to be euthanased.</td>
</tr>
</tbody>
</table>
2.1.2 lipopolysaccharide (LPS) -induced rat inflammation model

LPS, an ingredient of the gram-negative bacterial wall, initiates an innate immune response to produce pro-inflammatory cytokines. These cytokines lead to symptoms of inflammation, such as swelling, redness and pain. The degree of inflammation depends on the dose of LPS and route of administration.

Protocol: rats were anaesthetised with urethane (see 2.1.3). LPS (1.65 mg/kg, 10 mg LPS dissolved in 1 ml sterilised saline) was injected into the dorsal aspect of the rat hind foot intradermally. Approximately 200 µl of arterial blood was collected before and 4 hours after LPS administration to measure cytokines for inflammation assessment. Measurements of lymphatic function were made by near infrared imaging (see 2.2) before LPS treatment and at 4 hours after which the rats were euthanized.

2.1.3 Anesthesia

All animals undergoing lymphatic near infrared imaging were anaesthetised by intraperitoneal (i.p.) injection of urethane (1g/kg, 0.25g urethane dissolved in 1 ml Phosphate-buffered saline solution). Phosphate-buffered saline solution (PBS, gibo by life technologies, Cat# 21600-010) was prepared dissolving 9.6g PBS powder in 1000 ml distilled water, and pH adjusted to 7.1 (0.2-0.3 units below desired pH of 7.35).

The animals that were shaved for lymphatic near infrared imaging were anaesthetised with 2 ~ 3% isoflurane at 0.8 ~ 1.0 L/min flow rate, delivered by nose cone.

2.2 Development of near infrared (NIR) imaging

2.2.1 NIR lymphatic imaging

NIR imaging was used, as it is a good technique to assess lymphatic function in vivo. It exhibits: low light absorption and scattering in biological tissues; minimal autofluorescence; deeper depth penetration due to the long wavelength fluorescent light; and excellent contrast/spatial resolution (Rao, Dragulescu-Andrasi et al. 2007).

NIR light penetrates deeply into tissues and is absorbed by Indocyanine Green (ICG), a fluorescent dye that we used. This dye is selectively taken up by lymphatic vessels and when stimulated by NIR light of wavelength 780 nm emits fluorescent light at 830 nm, as we recorded with a NIR bandpass filter of 830 nm (830.0 IF 10 0.5" diameter...
http://www.cheshireoptical.com or Thorlab, FBH850-40 - Premium, Cat# FBH850-40 used) and a CCD or high sensitivity EMCCD digital camera. Resultant images were captured in real time by computer and appropriate imaging software. Analysis of resultant movie files was made in both space and time to extract the contraction frequency and contraction wave velocity. The system was developed in three stages with components upgraded in each stage this greatly improving image quality (Table 2.2).

### Table 2.2: Key characteristics of stages used in development of NIR imaging

<table>
<thead>
<tr>
<th>Stages</th>
<th>Light source</th>
<th>Camera</th>
<th>NIR dye</th>
<th>Lymphatic vessels</th>
<th>Approach</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage 1</td>
<td>100 mW laser beam</td>
<td>CCD camera</td>
<td>ICG: BSA</td>
<td>Surgically exposed deep groin lymphatics</td>
<td>Invasive</td>
</tr>
<tr>
<td>Stage 2</td>
<td>250 mW laser beam scattered with concave lens</td>
<td>IR CCD camera</td>
<td>ICG: BSA</td>
<td>Surgically exposed deep groin lymphatics</td>
<td>Invasive</td>
</tr>
<tr>
<td>Stage 3</td>
<td>250 mW laser beam scattered with diffuser</td>
<td>EMCCD</td>
<td>ICG: BSA</td>
<td>Groin lymphatics or inguinal-to-axillary lymphatics</td>
<td>Non-invasive</td>
</tr>
</tbody>
</table>

#### 2.2.2 Light source

At the very beginning of the system development (stage 1), 780 nm 100 mW laser beam (approximate illumination density 100 mW/cm²) was used to excite the fluorophore (ICG) in surgically exposed deep groin lymphatic vessels imaged with a CCD camera. Stage 2 involved the use of a 780 nm 250 mW laser beam scattered with a biconcave lens that reduced the illumination intensity to < 70.7 mW/cm². The final improvement made (Stage 3) was to improve imaging sensitivity allowing a marked reduction in the light intensity and increase the illumination area using a diffuser (1" round 20 degree circle tophat diffuser).
2.2.3 Image detecting devices

Initially an ordinary CCD camera with an 830 nm NIR bandpass filter was tested in stage 1 development of the technique but the NIR lymphatic signal was of low quality (Fig. 2.1A). Stage 2 incorporated an improved infrared (IR) CCD Camera (Hitachi KP-M2RP 1/2" Near Infrared B/W), leading to better image quality (Fig. 2.1B). This allowed us to perform high-resolution invasive lymphatic imaging experiments on deep groin lymphatic vessels. The image quality was further improved in stage 3 by using an EMCCD camera (Andor, iXon Ultra 897). This improvement allowed imaging of lymphatic vessels through the skin allowing the experiments to be performed non-invasively (Fig. 2.2 and Fig. 2.3).

Figure 2.1: Deep groin lymphatic vessels

(A) Stage 1 (invasive): Image of ICG fluorescence recorded with a standard CCD camera from surgically exposed deep groin lymphatic vessels by illumination with a 10 mV, 780 nm laser beam and an 830 nm bandpass filter. The bright signal in the middle was considered to be a fold in the lymphatic vessels. (B) Stage 2 (invasive): The illumination system was altered to use a 250 mV laser scattered with a biconcave lens and a Hitachi infrared sensitive CCD camera was now used. The imaging area and other components were as for Stage 1. The direction of lymph flow is indicated with arrow (note increased resolution).
Figure 2.2: Custom-made near infrared (NIR) imaging system

Stage 3 NIR imaging system used for our experiments. The rat was placed on the heating pad. Oxygen was provided through a mask. The NIR laser was spread by a diffuser over the groin imaging area with the emission light from the dye filtered by an 830 nm bandpass filter and detected by an EMCCD camera. The images were recorded using imaging workbench software (Indec Biosystems, Ca)
In stage 3, an EMCCD (*electron-multiplying CCD*) replaced the CCD allowing low-level detection of emission signals. The image was recorded through the shaved skin without surgery. The 250 mW laser beam further scattered with a tophat diffuser in order to expand the illumination and reduce the laser intensity. (A) Sample image showing right and left groin lymphatic vessels. (B) Inguinal-to-axillary lymphatic vessel (arrow shows lymph flow direction for both A and B).
2.2.4 Fluorescent dye for NIR imaging

2.2.4.1 Indocyanine green (ICG)

ICG is a US food and drug administration approved diagnostic dye and has been used safely in humans over last two decades for assessment in hepatic clearance (Ott, Keiding et al. 1992, Shinohara, Tanaka et al. 1996, Kortgen, Paxian et al. 2009) and diabetic retinopathy (Shiragami, Shiraga et al. 2002). It was first used in 1969 (Kogure and Choromokos 1969), and brought into clinical practice in 1992 (Yannuzzi, Slakter et al. 1992). NIR lymphatic vessel imaging with ICG also has been used for over ten years. ICG was used to map sentinel lymph nodes (SLNs) in human patients (Motomura, Inaji et al. 1999) and in a rat model (Kim, Song et al. 2010). Recently, especially in last five years, it has been used to assess lymphatic function in humans (Yamamoto, Narushima et al. 2011) and animals (Aldrich, Davies-Venn et al. 2012, Weiler, Kassis et al. 2012). The common use of ICG including its chemical stability and safety predicated its use in the NIR lymphatic experiments of this thesis.

2.2.4.2 The safety of ICG

Gashev et al. have expressed concern about the inertness of ICG in lymphatic experiments, as they found that irradiated ICG perfused in vitro slowed rat mesenteric lymphatic vessels (Gashev, Nagai et al. 2010). However, it is unclear how the concentration they used in vitro compares to the lymphatic concentration in vivo. In contrast, Aldrich et al. presented evidence that mouse inguinal-to-axillary lymphatic vessels studied in vivo did not exhibit inhibition when lymphatic imaging was performed with various concentrations of ICG (without albumin) injected into the tail base (Aldrich, Davies-Venn et al. 2012). This has also been supported by other studies where it was concluded that ICG did not change lymphatic function over the 20 minute recording period in which they made their initial study (Weiler, Kassis et al. 2012). Their subsequent study showed that ICG retained in tail lymphatics for ~ 2 weeks caused enlargement of lymph nodes and reduced lymphatic pumping but this recovered there being no significant difference from control after 1 month follow up (Weiler and Dixon 2013). Based on these research results, we considered ICG as appropriate to use in our short duration experiments.
2.2.4.3 Preparation of ICG solution for injection

Various ICG and albumin mixtures have been used in NIR human and animal lymphatic imaging. A concentration of 250 µg/ml ICG was used in breast lymphatic imaging in breast cancer patients (Sevick-Muraca, Sharma et al. 2008). Weiler et al. demonstrated that 150 µg/ml ICG in albumin (60 g/l) physiological salt solution (APSS) gave optimal results for rat-tail lymphatic imaging (Weiler, Kassis et al. 2012). Aldrich et al. used 0.32 ~ 1.3 mM ICG in mice inguinal-to-axillary lymphatic imaging, with lymphatic pumping not altered by this range of ICG concentrations (Aldrich, Davies-Venn et al. 2012). Some studies suggested that fluorescence intensity is enhanced when ICG was premixed with albumin (Sauda, Imasaka et al. 1986, Moody, Viskari et al. 1999). Various concentrations of ICG premixed with human serum albumin at different ratios indicated that 1:1 molar ratio produced the highest fluorescence intensity (Ohnishi, Lomnes et al. 2005). Based on these studies and pilot data we used 0.8 mM concentration of ICG premixed with BSA (1:1 molar ratio) in PBS solution for stages 2 and 3 of our studies.

2.2.4.4 Injection methods

Subcutaneous, intraplantar and intradermal administrations were utilised in our studies. In stages 1 and 2 of NIR development, intraplantar injection of ICG into the rat right food pad was used to mimic previous ink transit experiments performed in our lab (Saul et al., 2011), with lymphatic vessels in the deep groin area successfully visualised. In contrast, subcutaneous ICG injection into the base of rat-tail did not allow clear visualisation of groin lymphatic vessels even with stage 3 imaging. Therefore intradermal injection into the base of rat-tail was tested with clear NIR images of groin (See Fig. 6.1) and inguinal-to-axillary lymphatic vessels now obtained. This was presumably because of the dense network of initial lymphatics located in the dermis (Fig. 2.4) (Robinson, Kwon et al. 2013), with the fluorescent dye rapidly taken up by the initial lymphatics and subsequently transported to the collecting lymphatics (Fig. 2.4C). A volume of 0.3 ml (Unno, Nishiyama et al. 2008) and 10 µl (Robinson, Kwon et al. 2013) of ICG solution was reported to be injected in the human and mouse studies respectively. Based on the size of rat skin thickness, 50 µl of 0.8 mM ICG (premixed with BSA (1:1 molar ratio) solution was applied through intraplant or intradermal injection in all our rat experiments.
Figure 2.4: Schematic of initial lymphatics located in the dermis (A, modified from (Robinson, Kwon et al. 2013)). When ICG was injected into the mouse-tail base subcutaneously, lymphatic vessels could not be visualised even with an imaging system similar to our Stage 3 imaging (B). Clear and rapid visualisation was obtained when the ICG was injected intradermally (C).
2.2.5 Analysis of the NIR lymphatic images

First arrival time, contraction frequency and contraction wave velocity were extracted from the video images.

2.2.5.1 First arrival time: ICG was injected into the rat footpad, and image recording started immediately on surgically exposed deep groin lymphatic vessels. The images were loaded into ImageJ software (developed at National Institutes of Health, USA) for fluorescence intensity measurement over time. The first arrival time was determined as the time when fluorescence intensity in the deep groin lymphatic vessels had increased by 5% (Fig. 2.5). This measurement was only made for the experiments performed in the stage 2 experiments (see Chapter 3).

![Figure 2.5: First arrival time of ICG in groin lymphatic vessels](image)

(A) The first arrival time of the ICG was taken at the time point when the NIR ICG signal intensity had risen to 5%. (B) The expanded plot shows the first arrival time with an arrow, which in this case occurred 4.7 min after hind footpad injection.

2.2.5.2 Contraction frequency: Lymphatic contractions produce fluctuations in fluorescence intensity in a region of interest (ROI), and these fluctuations present as peaks when the fluorescence intensity is plotted over time. The fluorescence intensity increased when the lymphatic vessel dilated (Fig. 2.6A and Fig. 2.7D), and fluorescence intensity decreased when the vessel constricted (Fig 2.6B and Fig. 2.7E). The contraction frequency was calculated by counting the peaks per minute (Fig 2.6C, Fig 2.7A and Fig. 2.7B).
Figure 2.6: Measurement of deep groin lymphatic contraction frequency

The lymphatic contraction cycle includes dilation (A) and constriction (B) of the lymphangions as shown for the vessel section in the marked region of interest. (C) The contraction cycle measured by monitoring fluorescence intensity plotted against time. The contraction frequency was obtained by counting the fluorescent peaks. During 3 – 5 minute recordings. The contraction frequency in C was 5.5 contractions/min.
Figure 2.7: Measurement of contraction frequency in a groin lymphatic vessel (non-invasive).

(A) 10-min contraction plot of a groin lymphatic vessel measured non-invasively through the skin using the stage 3 imaging procedure. (B) Expanded plot for the first 100s of A showing positive and negative peaks. (C, D and E) show three different phases of a lymphatic vessel during the contraction cycle, namely: the diastolic phase; the dilated phase and the systolic (i.e. constricted) phase.
2.2.5.3 Contraction wave velocity: The serial lymphangions along the collecting lymphatics constrict to form contraction waves, which propagate at certain speeds. The contraction wave velocity was calculated with in-house Matlab-integrated software. Briefly, one pixel wide freehand ROIs were drawn (Fig. 2.8A). Time series traces for fluorescence intensity were generated for the chosen time period and a spatio-temporal map generated (Fig. 2.8B). From this spatio-temporal map contraction wave velocity was calculated by marking two points \((X_1, Y_1)\) and \((X_2, Y_2)\) where X represents time in seconds and Y spatial pixel location. Velocity was then calculated as \(V = \frac{L}{t}\) (pixels/s) with \(L = Y_2 - Y_1\) (pixels) and \(t = X_2 - X_1\) (s) where \(V\)=velocity, \(L\)=length (pixels) of the measured lymphatic vessel and \(t\)=time (s). Final velocity in cm/s was calculated by calibrating the inter pixel distance in cm using a graticule.
Figure 2.8: Analysis of contraction wave velocity

(A) Continuous distal-proximal ROI selection along the lymphatic vessel (length=2.35 cm). (B) Lymphatic flow spatio-temporal map. The direction of forward sequential contraction (i.e. distal-proximal) is indicated by the relative higher pixel intensities. The direction of backward sequential contraction (i.e. proximal-distal) is seen as relatively lower pixel intensities (see Chapter 5). Contraction velocity is calculated from the start \((X_1, Y_1)\) and end point \((X_2, Y_2)\) values.
2.2.5.4 5-minute traveling distance of contraction waves: This parameter was newly added to those parameters above to further understand lymphatic function.Normally, contraction waves initiate from the distal end of the collecting lymphatics and finish at a proximal point (forward contraction waves). The traveling distance of individual contractions may vary, depending upon many factors. Retrograde contraction waves were also observed, but only the forward contraction waves contribute to the propulsion of lymph fluid. The traveling distance within a certain time correlates to the volume of the lymph propelled within that particular time. The 5-minute contraction wave travelling distance calculated by adding up all individual travel distances of contraction waves within 5 minutes \((L_5)\) over the same vessel segment and calculated by: \(L_5 = a+b+….\) Where \(a, b….\) represent the distances the contraction wave travelled with each contraction (Fig. 2.9).

![Figure 2.9: Measurement of 5-minute travelling distance of contraction waves](image)

(A) Line used for producing the spatio-temporal map in B (direction is from distal to proximal – i.e. foot to body). (B) spatio-temporal map: Lymph contraction wave velocity \((V)\) and 5-minute contraction wave travel distance \((L_5)\) were calculated by measuring the summed distance that individual contraction waves travel when measured over a 5 minute period for the same vessel segment. Here \(V = L/t\) \((V\)-velocity, \(L\)-length, \(t\)-time and \(L_5 = a + b….\) where the lower-case letters represent the length that individual contraction waves travel).
2.3 Intra-arterial blood pressure (IABP) measurement

IABP measurement provides accurate and real-time values of arterial pressure in rats. In our studies, the rat blood pressure was measured via the carotid artery.

2.3.1 Measurement of IABP

2.3.1.1 Intra-arterial cannula

IABP measurement first involved cannulating the left carotid artery after the rat had become deeply anaesthetised. Arterial cannulation involved carefully exposing the left carotid artery and then ligating the distal end with cotton thread and clamping the proximal end. A small incision (v-shaped cut, 45°) was made with spring-scissors on the upper surface of the artery. A 15 cm long polyethylene tube (I.D. 0.58 mm, O.D. 0.96 mm, Dural Plastics & Engineering, Cat# 112031) connected to a 10 ml syringe (Terumo) and filled with 50 IU/ml heparin was inserted, and pushed slowly ~2 cm towards the main artery with the proximal artery then ligated with cotton thread to fix the cannula. The tube was flushed with 1 ml of heparin (50 IU/ml) after cannulation. Blood pressure was continuously recorded on a PowerLab 4/30 system (AD Instruments, Australia).

2.3.1.2 Arterial tube

The length and properties of the arterial tube has a large effect on damping. Damping refers to the factors that reduce the energy in an oscillating system leading to a decrease the amplitude of oscillations. Some damping is needed in blood pressure measurement in this system. The damping coefficient indicates the degree of damping, the optimal damping coefficient being about 0.7. Figure 2.10 shows how the damping coefficient is calculated. There are a number of factors contributing to damping, such as tube diameter and length, bubbles and clots, tube connections and 3-way tap (Abby Jones 2009). To obtain optimal damping, a 15 cm long polyethylene tube (non-compliant) is used for delivering the pressure waves to the transducer. Constant pressurised saline infusion at a rate of about 1 ml/h was used to prevent clotting.
The fast flush test is performed first by quickly squeezing the flush system to obtain a blood pressure wave as shown in this figure. And the amplitude ratio is calculated by dividing wave-component amplitudes A1 by A2 with the corresponding damping co-efficient looked up on the chart. Establishment of an optimised co-efficient improves the accuracy of the IABP measurement.

2.3.2 Data acquisition and analysis
The Powerlab and Bridge Amp were the main devices to convert the analog signal to numerical data and the numerical data were recorded and analysed with Labchart 7 (ADInstrument, Sydney Australia). Systolic, diastolic and mean arterial pressure (MAP), and heart rate (HR) values were extracted from raw pressure data. MAP is calculated as \[ MAP = \left( \frac{2 \times \text{diastolic} + \text{systolic}}{3} \right). \]
Chapter 3. Lymphatic Function Remains Robust in an Acute Diabetic Rat Model

3.1 Introduction

The lymphatic system forms part of the circulatory system. Lymphatic vessels are present as a dense network, paralleling that of blood vessels, throughout most of the body (Casley-Smith 1983). A primary function of the lymphatic system is to collect excess fluid from the interstitial space and transport it into the circulation to maintain homeostasis. Other roles include transport of immune cells and absorption of lipids from the intestine (Casley-Smith 1962, Witte, Way et al. 1997).

Diabetes has deleterious effects on peripheral tissues including lymphatic vessels, such as increased lymphatic vessel density by type 2 diabetes (Haemmerle, Keller et al. 2013). Cameron & Cotter reported an alteration in superior cervical ganglion perfusion, as early as 1 week following diabetes induced by streptozotocin (STZ) in rats (Cameron and Cotter 2001). Previously Feingold et al. reported that 24-hour lymphatic drainage increased four-fold in the STZ-induced diabetic rat model (Feingold, Zsigmond et al. 1985). Moriguchi et al. also reported a similar change in lymph flow volume in an alloxan-induced diabetic rat model. This increase in lymph flow continued for 60 days after alloxan treatment (Moriguchi, Sannomiya et al. 2005).

Diabetes is a complex disease where the primary insult, namely unregulated glucose levels, can lead to many secondary pathologies. Determining the time-course of these pathological changes following the onset of diabetes has important implications for therapeutic interventions. In vivo models allow us to map this time-course.

In this study we investigated the effects of factors such as hyperglycaemia on leg lymphatic function in early stage STZ-treated diabetic rats before there were longer-term diabetes-associated effects. We were particularly interested in this lymphatic pathway, as diabetes-associated circulatory problems are generally much worse in the legs and feet leading to diabetic foot syndrome, a condition that in humans often leads to amputation of toe, foot or leg. Specifically, we tested the hypothesis that a
consequence of acute STZ-induced diabetes would be impaired leg lymphatic function.

Lymphatic function was measured using near infrared (NIR) imaging (Unno, Nishiyama et al. 2008, Aldrich, Davies-Venn et al. 2012); a minimally invasive method used in sentinel lymph node mapping (Kim, Lim et al. 2004, Song, Kim et al. 2009, Erpelding, Kim et al. 2010, Pan, Pramanik et al. 2010) and in surgery (Tanaka, Ohnishi et al. 2007, Ashitate, Tanaka et al. 2011) to locate specific lymph node or lymphatic vessels.

### 3.2 Methods and Materials

#### 3.2.1 Animals

All animal protocols and procedures were approved by The University of Newcastle Animal Care and Ethics Committee (approval: A-2009-153). 9-13 weeks old adult male outbred Wistar rats were purchased from the Central Animal Service Unit of the University of Newcastle. All animals were housed for at least 7 days in the animal facility with a 12 hour light/dark cycle before induction of diabetes. Leg lymphatic function in the type 1 diabetes mellitus model (T1DM) was examined. The initial experiments first involved developing and testing the model for diabetic status (12 rats, body weight 328 ± 81 g). Lymphatic NIR imaging was then performed (5 diabetic rats, body weight 360 ± 94 g, 8 control rats 408 ± 59 g).

#### 3.2.2 Experimental design and type 1 diabetes model

The well-characterized STZ-induced T1DM model was used in this study (Gallagher, Liu et al. 2007, Searls, Loganathan et al. 2010, Miao, Niu et al. 2012) as described in Chapter 2. Briefly, T1DM was induced by single dose intraperitoneal injection of STZ solution. Diabetes was generally induced within about 24h as evidenced by the onset of hyperglycaemia, with those above 12 mmol/l deemed diabetic. Control animals received citrate buffer only. Food and water access were ad libitum and the cage bedding was changed two times a day. Blood glucose, urine ketones and body weight were monitored on a daily basis, with all values and observations recorded on the monitoring sheets and scored.
3.2.3 Monitoring the blood glucose level

This procedure was performed by restraining the rats in an approved restraining bag. The tail was exposed and dipped into warm water in order to dilate the tail vein. Blood (10-20 µl) was drawn carefully with 1 ml syringe with 26 gage needle. Blood glucose values were determined by sampling the blood using a glucose monitoring system (ACCU-CHEK Active Blood Glucose Monitoring System).

3.2.4 Exposing groin lymphatic vessels

Rats were anesthetized by intraperitoneal injection of urethane (1-1.5 g/kg, 0.25 g in 1 ml phosphate-buffered saline solution (PBS)), and leg and lower abdominal hair was removed with an electric shaver. Each anaesthetized rat was positioned on its back under a dissecting microscope (Olympus SZX12). A 3-4 cm incision was made in between the middle line and the anterior superior iliac spine, parallel to the middle line and muscles were separated in the thigh until the deep groin vessel cluster was seen.

After surgery, the anaesthetised rat was moved into the NIR imaging chamber, and placed on a heating pad with an anal temperature probe inserted (the heating mat with temperature controller was made by the University of Newcastle Health workshop). The deep groin vessel bundle was revealed for recording (Fig. 3.1 A) by pulling back the surrounding skin and muscles using fine hooks connected by fine nylon ties to a post. The connective tissue membrane that covered the vessel bundle was removed to improve NIR imaging of the lymphatic vessels. The incision was kept moist with PBS soaked gauze until commencement of the recording. The same procedure was repeated on the other side. Body temperature was maintained at 36 ~ 37.5°C with leg temperature maintained at 34°C by continuously pumping warm water through silicone tubing loosely wrapped around the rat leg. The leg temperature was monitored with a needle microprobe thermocouple (MT-29/1, ADInstrument Australia) inserted subcutaneously at the ankle. The pulse was monitored by an animal oximeter pod (Product# ML325/AC, ADInstrument Australia).
Figure 3.1: Deep groin lymphatic vessels visualised with ICG and near infrared (NIR) illumination

(A) Image of groin vascular bundle taken before injection of ICG. The vein is indicated by the arrow (under bright light). (B) Weak fluorescent signal in the vein observed ~30s after ICG injection before lymphatic visualisation. (C) Image of groin vascular bundle taken ~ 10 min after injection of ICG revealing 2-3 lymphatic vessels alongside the vein.

The rat was then stabilized on the heating pad for at least 1-hour before commencement of NIR recording. An IR video camera was positioned by focusing on a piece of silicone tube (length 1.5 cm; inside diameter 0.25 mm) filled with Indocyanine green: BSA solution (ICG: BSA; see below). The tube was placed in the incision parallel to the groin vein with the IR laser pre-set to cover the entire vessel bundle. Video recording was made one side at a time with experiments performed inside a laser safe hood.

3.2.5 Near Infrared Imaging

Rats were prepared for NIR lymphatic imaging 3-5 days after STZ treatment. The deep groin vessel bundle was exposed (see above) and NIR lymphatic imaging performed by intraplantar injection of 50 µl ICG: BSA into the foot. The right and left lymphatic vessel images were recorded separately for up to 35 minutes for each side (Fig. 3.1C).
Our customized near infrared imaging system used a 250 mW, 780nm laser diode and biconcave lens (illumination density < 70.7 mW/cm²) mounted in a Thorlab cage system. Images were captured using a Hitachi KP-M2RP 1/2" Near Infrared B/W CCD Camera. A near infrared bandpass filter (830.0 IF 10 0.5" diameter http://www.cheshireoptical.com) and a Fuji DV10x7B-2 camera lens was used. Videos were recorded with a VHS to DVD 4.0 converter (Honestech) linked to a Dell Latitude E6330 laptop computer. Recording was made in an enclosed lightproof chamber with cut-off switch (Fig. 3.2).

![Diagram](image)

Figure 3.2: Near infrared (NIR) lymphatic imaging (stage 2)

ICG injected into a rat hind foot is taken up by the lymphatics and passes through the deep groin lymphatics. The 780 nm wavelength NIR light from the laser diode is absorbed by ICG causing fluorescence emitted at 830 nm, which is detected by a CCD camera after passing through an 830 nm bandpass filter. Resultant video images were captured on a computer with VHS to DVD converting software. Laser safety was ensured by enclosing the rat and illuminating/video system in a lightproof cage.

### 3.2.5.1 Indocyanine green-bovine serum albumin solution (ICG: BSA)

ICG and bovine serum albumin (BSA) were purchased from Sigma-Aldrich (ICG Cat# I2633, BSA Cat# A4503). BSA powder (106 mg) was dissolved in 1.5 ml PBS with ICG (0.62 mg/mL) then dissolved in 1 ml of the PBS-BSA solution, mixed for
approximately 30 seconds in a vortex shaker and stored in the dark. The final concentration of the solution was 0.8 mM, ICG: BSA molar ratio is 1:1. The ICG solution was used within 8 hours.

3.2.5.2 Analysis: The video file was analysed using ImageJ (developed at National Institutes of Health, USA). A region of interest (ROI) was selected and its intensity was measured throughout the video recording (approximately 30 minutes). The values were plotted against time in Matlab with the time at which a 5% increase in signal intensity was reached noted as the arrival time (See chapter 2 Fig. 2.5). Contraction frequency was calculated by counting the signal peaks after maximal signal was reached (See chapter 2 Fig. 2.6).

3.2.6 Statistical analysis:
Data were analysed using MS Excel 2013 or Prism 6 (Graphpad, USA). All data were tested with the Kolmogorov–Smirnov normality test and D'Agostino & Pearson omnibus normality test. Significance of the first arrival time and contraction frequency were determined using parametric Student’s t test for Gaussian distributed data and Mann–Whitney test for non-Gaussian distributed data. A paired t test was used for blood glucose comparison. The Friedman test (non-parametric, repeated measures) was used for body weight loss analysis. Values are expressed as mean ± SD as noted, with n referring to the number of animals used.

3.3 Results
3.3.1 Type 1 diabetic model assessment
The T1DM model caused by injection of 65 mg/kg STZ (i.p.) induced a diabetogenic rate of 83.3% within 24 hours and 100% in 48 hours. The blood glucose level was elevated from 7.0 ± 0.4 to 17.8 ± 4.8 mmol/l in the first 24 hours after STZ-treatment, an increase of 154% (n=12, P<0.0001, Fig. 3.3A). A significant body weight loss was observed within the first 3 days of diabetic induction, the average accumulative body weight loss being 6.3% (Day1), 9.6% (Day2) and 8.8% (Day3) (Fig. 3.3B, P < 0.001) with body weight then generally remaining constant or gradually increasing. A small percentage of rats (16.6%) exhibited severe symptoms and were euthanized (see 2.1.1 for criteria).
3.3.2 **NIR deep groin lymphatic imaging**

The femoral vein, artery and lymphatic vessels were located side by side in the groin area. There was a weak NIR signal from the vein, which appeared within 10s of the injection of the ICG: BSA solution into the foot (Fig. 3.1B). The NIR signal in lymphatic vessels was visualised much later than this, taking about 5 min (see below). Two to three branches of lymphatic vessels were visualised alongside the vascular bundle in the deep groin area (Fig. 3.1C). NIR imaging enabled us to observe and quantify lymphatic chamber activity. The first arrival time and contraction frequency were extracted from the video recordings. It was, however, difficult to extract information on contraction amplitude due to false increases of true diameter of the vessel through NIR signal emission spread.

![Figure 3.3: STZ-treatment induced blood glucose and weight loss](image)

**Figure 3.3: STZ-treatment induced blood glucose and weight loss**

(A) Blood glucose was elevated significantly, within 24 hours, by STZ-induced destruction of pancreatic β cells (Student’s t test, expressed as mean ± SD, n=12, \( P<0.0001 \)). (B) Rats lost considerable body weight (normalised wt/wt\(_{Day0}\)) due to metabolic disturbances (Friedman test, expressed as mean ± SD, n=12, \( P<0.001 \)).

Contraction frequencies (Fig. 3.4A) in the control group were 6.4 ± 3.5 contractions/min (right, n=8) and 6.4 ± 2.7 contractions/min (left, n=6). The corresponding contraction frequencies of lymphatics in these STZ-treated rats were not significantly altered from control (\( P>0.05 \)) with values of 6.5 ± 4.3 contractions/min for the right side (n=6, \( P=0.9 \)) and 4.3 ± 2.3 contractions/min for the left side (n=5, \( P=0.5 \)).
The first arrival times of the NIR dye, which represents dye absorption by the initial lymphatics and transport velocity by the collecting lymphatics, were $6.7 \pm 3.2$ minutes for the right side ($n=5$) and $3.2 \pm 2.5$ minutes for the left side ($n=5$) (Fig. 3.4B). The corresponding first NIR dye arrival times in diabetic rats was $6.7 \pm 5.6$ minutes for the right side ($n=5$) and $4.4 \pm 3.1$ minutes for the left side ($n=5$). These values are not significantly different to the controls (right $P=0.9$, left $P=0.6$) indicating that this component of leg lymphatic function was not altered in the diabetic rats despite the animals exhibiting substantial glucose levels (i.e. $27 \pm 5$ mmol/l measured over 3-5 days after STZ treatment).

![Figure 3.4](image)

**Figure 3.4: Contractile properties of groin lymphatic vessels in control and diabetic rats**

(A) Contraction frequencies measured by NIR imaging of ICG from both right and left side deep groin lymphatic vessels were not significantly different in diabetic compared to control rats (individual values plotted, right $P=0.9$, left $P=0.5$). (B) The first arrival time also did not exhibit significant differences between the control and diabetic group (individual values plotted, right $P=0.9$, left $P=0.6$).
3.4 Discussion

3.4.1 Type 1 diabetic rat model

There are many animal models of type 1 and type 2 used in diabetic research (i.e. chemically or nutritionally induced diabetes and genetically modified rat diabetes model). The streptozotocin (STZ) model, which destroys insulin-secreting pancreatic β cells in pancreatic islets is widely accepted to capture the main pathology in the acute phase. We used a dose of 65 mg/kg STZ, as this produced a 100% diabetogenic effect with low.

3.4.2 Type 1 diabetes and lymphatic contractile properties

There are a limited number of studies on the effects of type 1 diabetes on lymphatic contraction. Lymph volume was increased in 10, 30, and 60-day diabetic rats and abnormal lymphocyte output from thoracic duct was observed in the first 10 days of diabetes (Moriguchi, Sannomiya et al. 2005). The augmented lymph volume observed in their studies was most likely due to both an increase in contraction frequency and/or an increase in stroke volume (Benoit, Zawieja et al. 1989). Such increases depend on increased production of lymph, a process in which increased glucose levels could be a driving factor. For example, noting that the glucose concentration in whole blood is ~15% lower than in serum (McMillin 1990), then the blood glucose levels in the diabetic rats of our study of ~18 mmol/l correspond to an average serum glucose level of 20 mmol/l. In rats, the glucose concentration in lymph fluid is higher than in serum, the ratio being 1.08 (Rasio, Soeldner et al. 1965). Based on this, we estimate the lymph glucose concentration in our diabetic rats to be near 22 mmol/l. This concentration could contribute to enhanced lymph formation due to increased osmotic pressure. A higher volume of lymph fluid should increase the distension pressure of the lymphatic vessel wall, this increasing stroke volume. However, initial increased distension by luminal pressure generally leads to increased frequency of contractions, but further increase could lead to a resultant fall in phasic contraction frequency and strength (Zawieja 2009) (see Fig. 3.4B).

In our study of acute phase of type 1 diabetes, we found the lymphatic function (i.e. lymph arrival time and lymphatic contraction frequency) remained rather robust in spite of high blood glucose. Increased glucose in the short term has no negative impact on lymphatic function. This suggests that long-term changes in oedema and other
lymphatic-associated pathologies are secondary to glucose imbalance. This said it remains surprising that there was not an increase in contraction frequency given the expected increase in lymph formation suggesting that glucose itself may have a small inhibitory effect on lymphatic activity.

Some variance was noted in contraction frequency and first arrival time in both the control and diabetes groups, with age variation of the animals considered a likely cause (Liao, Jones et al. 2014).

3.5 Conclusions
The type 1 diabetes mellitus rat model induced with 65 mg/kg STZ demonstrated a large diabetogenic effect with a significant acute increase in blood glucose concentration from 7 to 18 mmol/l one day after treatment. Contrary to our hypothesis, we found that lymphatic function remained robust. The long-term effects of type 1 diabetes on lymphatic function need to be investigated.
Chapter 4. Consequences of Glibenclamide on Wound Healing in an Acute Diabetic Rat Model

4.1 Introduction

Glibenclamide is a widely prescribed hypoglycaemic drug (sulfonylurea) for type 2 diabetes worldwide. It improves glucose control by secretion of insulin from the pancreas (Lebovitz, Feinglos et al. 1977, Kolterman, Gray et al. 1984, Simonson, Ferrannini et al. 1984). However, a potential complication associated with the use of glibenclamide is its reported effects on wound healing having been shown to inhibit this process in cases such as open skin wound in rabbits (Pipelzadeh, Pipelzadeh et al. 2003), indomethacin-induced gastric ulceration in rats (Toroudi, Rahgozar et al. 1999) and alveolar epithelial wounds (Trinh, Prive et al. 2007). This is of considerable concern given that glibenclamide is used to help in glucose control for patients with type 2 diabetes, yet diabetic foot ulcers are a common complication in poorly controlled diabetic patients. Foot ulcers occur in 15% of all diabetic patients and their poor healing underlying approximately 84% of all amputations (Pecoraro, Reiber et al. 1990).

The effects of glibenclamide on wound healing under hyperglycaemic conditions are poorly understood. Clearly, complications associated with hyperglycaemia are themselves a major negative factor in wound healing with healing impaired in both type 1 and type 2 diabetes, as shown in human (Moulik, Mtonga et al. 2003) and animal models (Goodson and Hunt 1979, Seifter, Rettura et al. 1981). Hence determining whether glibenclamide further exacerbates this is important, as should glibenclamide together with hyperglycaemia exert a synergistic adverse effect on wound healing, then its usage should be carefully considered. This said, its actions on diabetic wound healing could be beneficial, not only in its capacity to reduce blood glucose by inhibiting $K_{ATP}$ channels on pancreatic $\beta$ cells in type 2 diabetes, but by inhibiting inflammation-activated $K_{ATP}$ channels in other cells including those in lymphatic smooth muscle (Mathias and von der Weid 2013). Inhibition of $K_{ATP}$ channels in these vessels could enhance wound healing by factors improving circulation of immune competent cells and removal of cellular debris. $K_{ATP}$ channels are also enhanced in macrophages under inflammatory conditions (Ling, Ma et al. 2013), such as may be induced in hyperglycaemic conditions that occur in poorly controlled diabetes. The
direct consequences of glibenclamide action are therefore uncertain and need to be
investigated under hyperglycaemic conditions preferably where confounding effects
caused by its well-known action on pancreatic $\beta$ cells are substantially decreased.

The experiments reported here tested the hypothesis that wound healing in a rat model
of acute STZ-induced diabetes can be improved by blocking lymphatic $K_{ATP}$ channels.
Specifically we measured the effects of $K_{ATP}$ channel blockade on hind foot wound
healing in a streptozotocin (STZ) -induced hyperglycaemic rat model of type 1 diabetes
caused through toxicity of STZ on pancreatic $\beta$ cells.

4.2 Methods and Materials

4.2.1 Animals
All animal protocols and procedures were approved by The University of Newcastle
Animal Care and Ethics Committee (Approval: A-2009-153). Wistar outbred rats
(weight 362 $\pm$ 37 g, age 9-13 weeks, n=17) were bred and housed by the Animal
Services Unit at the University of Newcastle.

4.2.2 Experimental design
Animals were housed with 2 rats in each cage in the animal holding facility for 7 days.
Type 1 diabetes mellitus (T1DM) was then induced. Briefly, 65 mg/kg STZ was
injected intraperitoneally in rats in the diabetic group, which was then subdivided into
diabetic glibenclamide (DM-glibenclamide, n=6) and vehicle (DM-vehicle, n=5)
groups. Citrate buffer was administered in the control group (n=6). Hyperglycaemia
was confirmed the next day by measuring blood glucose levels and those with over 12
mmol/l of blood glucose deemed as diabetic. After 3 days induction of diabetes, a 5-6
mm diameter wound was created on the dorsal side of the right hind foot. Blood
glucose, weight and wound condition were monitored and a photograph of the wound
taken daily. Animal behaviour was monitored and scored according to the monitoring
checklist. Glibenclamide cream (6%) or vitamin E cream, the latter as vehicle, were
applied twice daily on the shaved right hind leg including near to but not on the wound.
Topical leg application, was used as we were specifically interested in the local effects
of glibenclamide on wound healing. Studies using other lipophilic substances indicate
such application is highly effective (Yamamoto, Katakabe et al. 1990, Saul, Thomas et
Rats with urine ketones, body weight loss \( \geq 20\% \), blood glucose > 33 mmol/l and/or severe illness (overall assessment score > 10 and manifested with tip-toe waking, comatose, unresponsive to extraneous activity or stimulation) were euthanized though these instances were rare. All rats that passed these tests were used for experimentation and euthanized at the end of the experiment when the wound was fully closed.

### 4.2.3 Open skin wound model (Fig. 4.1)

Rats were anesthetized with isoflurane after which the hair on the right leg was shaved with a pet clipper. The dorsal side of the foot was disinfected with 70% ethanol. Then, a circular patch of skin 5-6 mm in diameter was cut out with a disposable biopsy punch (5 and 6 mm in size, produced by the Kai Group and purchased from https://www.medshop.com.au). Photographs were then taken with a digital camera (Sony, cyber-shot DSC-W710, 16.1 mega pixels) and glibenclamide or vitamin E cream (as vehicle) was applied onto the right leg in the DM-glibenclamide and DM-vehicle groups respectively (see above). No cream was applied to a third control rat group. The pain-relieving drug Tramadol (5mg/kg) was administered intramuscularly on the day that the wound was inflicted and orally (10 mg/kg) twice in the following 3 days.

### 4.2.4 Glibenclamide cream

Glibenclamide (Cat# G0639-10G) was purchased from Sigma-Aldrich and vitamin E cream (INVITE, cream pump bottle 200g) was purchased from a local pharmacy. Glibenclamide (0.6 g) was evenly mixed with 9.4 g of vitamin E cream, until the drug particles dissolve completely, to form 6% (w/w) glibenclamide cream. It was stored at 4\(^\circ\)C and used within 2 days of preparation.

### 4.2.5 Analysis

Wound photographs were analysed using ImageJ (developed at National Institutes of Health, USA) with wound area and perimeter measured daily for each animal. The daily healing rate was expressed as the linear healing of the wound edge. It was calculated with Gilman’s equation as \( \Delta A / \bar{P} \), where \( \Delta A \) is the absolute area change between two consecutive days and \( \bar{P} \) is the average perimeter calculated from two perimeters measured in the same two consecutive days (Gilman 1990). The average daily healing
rate was obtained by the sum of the daily healing rates divided by the number of days to complete closure. The normalised wound area was obtained each day and the daily average normalised area per group calculated. The 20%, 50%, 80%, and complete wound closure times were obtained from these values by 4\textsuperscript{th} degree polynomial curve fitting (Matlab version: R2014b, the MathWorks, Inc., USA).

4.2.6 Statistical analysis

Statistical analysis was performed using Prism 6 (Graphpad, USA). Values are expressed as Mean ± SD with n referring to the number of animals used. Two-way ANOVA was used to compare the daily normalised wound area between groups and blood glucose comparison. One-way ANOVA was used for other comparisons (\( \alpha \) was defined as 0.05).

Figure 4.1: Open skin wound on day 1 and day 7

The upper photographs demonstrate the freshly created open skin wounds on day 1 in control, diabetes-glibenclamide and diabetes-vehicle treated rats. The lower images show the corresponding healing status on day 7. Hyperglycaemia had the most dominant effect in impairing wound healing.
4.3 Results

4.3.1 Blood glucose levels

Consequent to STZ treatment and after wound creation, the blood glucose elevated from a basal average of 6.8 ± 0.2 mmol/l to daily average of 22.4 ± 1.4 mmol/l in the DM-glibenclamide group (n=6) and from a basal average of 6.9 ± 0.2 mmol/l to a daily average of 18.9 ± 3.5 mmol/l in the DM-vehicle group (n=5). The daily average blood glucose after wound creation in the DM-glibenclamide group was higher than in the DM-vehicle group (P < 0.05) (Fig. 4.2).

![Figure 4.2: Blood glucose level comparison](image)

The post-treatment average blood glucose (BG) was obtained by averaging the blood glucose concentrations measured daily. The Streptozotocin treatment caused significant hyperglycaemia in both the DM-glibenclamide and DM-vehicle groups. Glibenclamide elevated the post-treatment average blood glucose significantly compared to post-treatment blood glucose in vehicle (P < 0.05). (Values are expressed as mean ± SD)

4.3.2 Wound assessment

STZ-induced diabetes caused weight loss and a small percentage of rats exhibited severe symptoms and were euthanized (see 2.1.1 for criteria). Although the shape of the circular, biopsy punch-induced skin wound changed daily during healing (Fig. 4.1), it remained possible to measure the area and perimeter of the wound precisely. The initial wound area in all groups ranged from 14 to 34 mm² with no significant differences found between groups in initial wound size (P > 0.99). Wound creation was successful in every rat with no complications (i.e. haemorrhage, tendon or nerve damage and infections) observed during the procedure or subsequently during healing.
The wound area for control, DM-glibenclamide and DM-vehicle groups demonstrated progressive reduction in wound size with time (Fig. 4.3). The diabetic group (with vehicle) was the first to exhibit a significant delay in wound healing (i.e. at the 3rd day) compared to control group ($P < 0.05$). On day 6, the normalised wound areas were 0.3, 0.7 and 0.6 for control, DM-glibenclamide and DM-vehicle groups respectively and both DM-glibenclamide and DM-vehicle groups presented significant differences in wound size compared to control ($P < 0.001$ and $P < 0.01$ respectively). This difference remained until day 7. The daily linear advance of the wound edge, calculated based on the area and perimeter was 0.27 mm/day, 0.21 mm/day and 0.23 mm/day in control, DM-glibenclamide and DM-vehicle (Fig. 4.4). The average DM-glibenclamide value was significantly different to that of control ($P < 0.05$).

![Daily Normalised Wound Area](Figure 4.3)

**Figure 4.3: Wound healing rates**

Wound healing rates expressed with daily-normalised wound area (WA) in the diabetic groups clearly separated from the control group as early as the 3rd day after wound creation. The difference between the diabetic and control groups was significant on day 6 and 7. The significance of the DM-glibenclamide group compared to control was higher (Values are expressed as mean ± SD).
Chapter 4

Figure 4.4: Linear advance of wound edge

The linear advance of the wound edge in the DM-glibenclamide group was significantly slower than control. The DM-vehicle group showed a similar trend but the change was not significant (Values are expressed as mean ± SD).

Wound assessment was also made by calculating the time taken for 20%, 50%, 80% and complete closure of wound (Fig. 4.5). No significant differences were observed for 20%, 50% and complete wound closure time points. The 80% wound area closure point healed over an average of 6.6, 9.6 and 9.4 days for the control, DM-glibenclamide and DM-vehicle groups respectively. The DM-glibenclamide group was the only one that presented significant differences compared to control ($P < 0.05$) at this closure point.
Figure 4.5: Wound area closing times

Significant differences were found for the 80% healing stage for DM-glibenclamide group compared to control group ($P < 0.05$). No significant differences were observed between control, DM-glibenclamide and DM-vehicle groups in early (A), middle (B) and late stage healing (D). (Values are expressed as mean ± SD)
4.4 Discussion

This study has investigated the effects of glibenclamide on wound healing in hyperglycaemic conditions. Glibenclamide, as a hypoglycaemic drug, blocks ATP-dependent potassium channels (K$_{ATP}$) in pancreatic $\beta$ cells to release insulin (Gerich 1989). In our rat model of Type 1 diabetes, this action will be muted because of substantial STZ-induced destruction of pancreatic $\beta$ cells.

We analysed wound healing rates by several methods, these being: the percentage wound area per day (compared to starting wound area); the average time for the wound to close to a specified percentage; the time to complete wound closure; and the linear advance of the wound edge. There was a variation in initial wound size in individual rats due to factors such as different skin tension, foot surface area and wound size, but no initial relationship was found that correlated to the hyperglycaemic state indicating that hyperglycaemia per se does not have an acute effect on skin mechanical properties. Generally we found that wound area expanded the day after wound preparation but started to shrink by the second day.

Assessment of wound healing using normalised wound area indicated there was a significant difference between control and DM-vehicle, confirming the previously reported adverse effect of hyperglycaemia on healing (Goodson and Hunt 1979). Glibenclamide made this adverse effect more significant on day 6 and 7 (Fig. 4.2A). The measurement of the advance of the wound edge demonstrated various daily healing rates in each group. The average linear advance of the wound edge, unlike the normalised wound area, did not show a significant difference between control and DM-vehicle though linear advance of the wound edge showed significant slow healing rate for the DM-glibenclamide group (Fig. 4.2B). The same result was obtained at the 80% closure time compared between each group (Fig. 4.3C). These results suggest that glibenclamide had a small additional adverse effect on wound healing in hyperglycaemia. A possible explanation is that the worsened outcome with glibenclamide resulted from the differential increase in hyperglycaemia, which while small was significantly increased compared to the DM-vehicle group. Thus the glibenclamide effect on wound healing in this type 1 rat model of diabetes under the
conditions of our study may simply arise through the higher glucose concentration and associated toxicity (Robertson, Harmon et al. 2003).

The glibenclamide-induced increase rather than decrease in glucose concentration deserves further consideration. While this increase is contrary to expectations given glibenclamide is normally hypoglycaemic, including as shown for topical application to non-diabetic rats (Yamamoto, Katakabe et al. 1990) such action will depend on the state of the pancreatic islet cells that in our STZ-treated rats may have been too damaged to respond. Other factors include: 1) the high local but comparatively low systemic concentration of glibenclamide, the latter likely to be the order of 400 ng/ml blood as found for topical application of 5% glibenclamide (Yamamoto, Katakabe et al. 1990) and 2) the finding that diabetes can cause decrease in mitochondrial \( K_{ATP} \) channels (Li, Huang et al. 2013). Glibenclamide would further inhibit these channels, mitochondria now generating negative influences that lead to the increase in glucose levels.

There are many physiological factors known to contribute to slow wound healing. These include impaired growth factor production (Falanga 2005, Galkowska, Wojewodzka et al. 2006, Goren, Muller et al. 2006), angiogenic factors (Galiano, Tepper et al. 2004, Falanga 2005), macrophage function (Maruyama, Asai et al. 2007) and many more. The hyperglycaemia induced by STZ is also known to decrease re-epithelialisation and delay wound healing (Velander, Theopold et al. 2008). As noted from wetting of the bedding, the diabetic rats produced more urine. This may lead to dehydration and electrolyte imbalance, and poor blood circulation to the wound area (Atchley, Loeb et al. 1933). Furthermore, high glycated haemoglobin increases the oxygen affinity to haemoglobin and hypoxia may ensue (Bunn, Gabbay et al. 1978, O'Riordan, Goldstick et al. 1984, Niwa, Naito et al. 2000). Other hypotheses for slow wound healing in hyperglycaemia include formation of glycation end products (Friedman 1999), hyperosmolarity (Yki-Jarvinen 1998) and altered insulin signalling (Porte and Schwartz 1996). Neuropathic and arterial complications due to long term damage by hyperglycaemia have not been considered in this short-term diabetic model.
4.5 Conclusions

Hyperglycaemia impaired and glibenclamide had a further small but significant additive adverse effect on foot wound healing in STZ-induced type 1 diabetic rats, which was contrary to our hypothesis. In the conditions of our experiment, glibenclamide caused a small but significant elevation in blood glucose and it is proposed that this differential increase underlies the further slowing of wound healing and not the glibenclamide itself.
Chapter 5. Investigating the effects of Pro-inflammatory Cytokines Induced by Moderate LPS Administration on Lymphatic Function

5.1 Introduction

Inflammation, a pathological process in many diseases and injuries, generally initiates an immune response, which in turn involves the lymphatic system in delivering immune cells (Witte, Bernas et al. 2001, Oliver and Detmar 2002). Therefore, maintenance of a functional lymphatic system under inflammatory pathological conditions is important and when compromised can contribute to the pathophysiology (e.g. in inflammatory bowel disease, IBD) (Heatley, Bolton et al. 1980).

Gram-negative bacteria are an important cause of infectious disease in humans. Severe infection may lead to life-threatening septic shock. The outer membrane of bacteria contains lipopolysaccharides (LPS), which initiate an innate immune response to produce pro-inflammatory cytokines. The effects of LPS on vascular smooth muscle have been well studied in inflammation and various septic shock models both in vivo and in vitro. LPS have a relaxant effect on vascular smooth muscle leading to a decrease in blood pressure (Wu, Thiemermann et al. 1995), which in the case of septic shock can be fatal (mortality rate in critically ill patients: 18.4% in Australia and New Zealand in 2012) (Kaukonen, Bailey et al. 2014). Importantly, studies on an animal model, the LPS-treated rat, indicate that glibenclamide administered during induction of sepsis in a rat septic shock model (Wu, Thiemermann et al. 1995) or in rats after 24-hour of LPS treatment (Sorrentino, d'Emmanuele di Villa Bianca et al. 1999) counteracts the decrease in blood pressure, suggesting sepsis-related upregulation of K\textsubscript{ATP} channels. Inflammation is known to upregulate K\textsubscript{ATP} channels as shown for mesenteric lymphatic smooth muscle where there is an upregulation of K\textsubscript{ATP} channels in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced ileitis (Mathias and von der Weid 2013).

LPS also interacts with lymphatic vessels during and after delivery of bacteria to lymph nodes and can modulate lymphatic function (Aldrich and Sevick-Muraca 2013). Specifically, when high doses of LPS were injected locally, lymphatic pumping
measured 4 hours after LPS injection was significantly inhibited in inguinal-to-axillary lymphatic vessels. Measurement of cytokine levels at this time showed them to be elevated. Local injection of other selected cytokines also induced inhibition of lymphatic pumping (Aldrich and Sevick-Muraca 2013). This latter study demonstrated the importance of cytokines as mediators in regulation of lymphatic pumping. The dose of LPS in their study caused a high level of inflammation. However, the effects on lymphatic function of doses of LPS that give moderate inflammation is not known and this forms the basis of the present study where we tested the hypothesis that low to moderate levels of inflammation impair lymphatic function. We were particularly interested in determining whether lymphatic contractile function (i.e. pumping) was inhibited and if so whether there was involvement of $K_{\text{ATP}}$ channels with function restored by $K_{\text{ATP}}$ blockers such as glibenclamide.

5.2 Methods and Materials

5.2.1 Animals

All animal protocols and procedures were approved by The University of Newcastle Animal Care and Ethics Committee (approval: A-2009-153). Wistar outbred rats (weight 265 ± 24 g, age 8-11 weeks) were bred by the Animal Services Unit at the University of Newcastle. Animals were divided into four groups, a saline (treated with saline, n=5), LPS (treated with LPS, n=5), LPS-glibenclamide (treated with LPS, and glibenclamide after 3 hours of LPS administration, n=6) and LPS-vehicle (treated with LPS, and DMSO as vehicle after 3 hours of LPS administration, n=5) group. Rats were acclimatised for 7-days in our animal holding facility under a 12:12 h light-dark regime prior to commencement of the experiment. Rats were anesthetized with isoflurane after which the hair on the right and left side of the abdomen and chest was shaved with a pet clipper and covered with hair-removal cream (Nair Dry & Sensitive, Church & Dwight, Sydney) for 5 min. The cream and loose hair was gently removed with a plastic scraper and the remaining cream rinsed off with warm water. The animals were then allowed to recover from the anaesthetic.

5.2.2 Experimental Protocol

Figure 5.1 shows the experimental design. Rats were shaved as described above. One or two days later, rats were deeply anaesthetized by urethane with intraperitoneal injection
(1g/kg in Phosphate-buffered saline: PBS 0.25g/ml), and placed on a temperature controlled heating pad with body temperature monitored by a rectal thermocouple (rectal temperature 37°C). The rats were stabilised for up to 30-minutes. Medical oxygen at 0.25 l/min was provided via a facemask throughout the experiment with oxygen levels monitored using an Animal Oximeter Pod (Model No.: ML325, AD Instruments, Australia) with the animal clip on the front paw and oxygen saturation maintained at 95~100%.

**Figure 5.1: Experimental design**

The shaved rat was anaesthetised by urethane (1g/kg) and stabilised for up to 30 mins. Baseline near infrared (NIR) images were recorded for 10 mins by administration of ICG (50 µl) on the right (R) and left (L) sides of the tail base respectively, with blood (200 µl) then rapidly collected from the tail artery for cytokine measurement. LPS or saline was administrated intradermally on the dorsal side of the right foot. Glibenclamide (40 mg/kg) or vehicle (0.5 ml/kg) was administered intraperitoneally after 1 hour of LPS treatment (this step were skipped for Saline and LPS group). NIR images were recorded and a 200 µl blood sample collected for cytokine measurement after 4 hours of LPS or Saline treatment. The rats were euthanized at the end of experiment (ICG-Indocyanine green/bovine serum albumin solution, REC-video recording, m-minute, Glibenclamide-G, Vehicle-V).
When the body temperature and oxygen saturation reached the desired level, two 10-minute near infrared video images were recorded for the right and left lymphatic vessels that traversed from the inguinal-to-axillary lymph node by injecting 50 µl ICG-BSA solution intradermally into the right and left sides of the base of the tail. Immediately after imaging, 200 µl blood was drawn from tail artery for measuring baseline cytokine/chemokine levels and the site of blood collection then carefully monitored to ensure bleeding had stopped. After this saline (saline group) or LPS at 1.65 mg/kg (LPS, LPS-glibenclamide, LPS-vehicle groups) was injected intradermally into the dorsal skin of right foot. The LPS-glibenclamide and LPS-vehicle groups differed to the LPS group in that glibenclamide (40 mg/kg in DMSO) or the corresponding volume of vehicle (0.5 ml/kg DMSO) was injected into the intraperitoneal cavity (i.p.) 1 hour after the LPS injection. Repeat NIR imaging was performed on the right and left sides of each rat in the same manner 3 hours after LPS injection with 200 µl blood then drawn for post-treatment cytokine measurement. The anaesthetized rats were euthanized after this step in the experiment.

5.2.3 Reagents

5.2.3.1 Indocyanine green (ICG)-bovine serum albumin (BSA) solution: ICG and BSA were purchased from Sigma-Aldrich (ICG Cat# I2633, BSA Cat# A4503). ICG was made up to a final concentration of 0.8 mM (0.62 mg/ml) in physiological buffered saline (PBS) containing 53 mg/ml BSA (ICG: BSA ratio 1:1). The ICG-BSA solution was stored in the dark and used within 8h of preparation.

5.2.3.2 Lipopolysaccharide (LPS) solution

LPS was purchased from Sigma-Aldrich (#L 3024, E. coli serotype 0111:B4), with 10 mg LPS dissolved in 1 ml sterilized saline (Pfizer, 0.9% sodium chloride injection, Cat# 19042010) to make a final stock solution concentration of 10 mg/ml, which was then pipetted into eppendorf tubes at 50 µl/tube and frozen until use. LPS (400 µg; 40 µl) was injected in each animal, any leftover LPS solution after thaw was discarded. LPS from E. coli serotype 0111:B4 was more potent compared to 026:B6 (Watanabe and Jaffe 1993), and it was also used by mice foot inflammation model to investigate lymphatic pumping (Aldrich and Sevick-Muraca 2013).
5.2.3.3 Glibenclamide solution
Glibenclamide (Cat# G0639-10G) and dimethyl sulphoxide (DMSO, Cat# D5879-1L) were purchased from Sigma-Aldrich. Glibenclamide, dissolved in DMSO at 80 mg/ml, was injected i.p. at 40 mg/kg (Sorrentino, d'Emmanuele di Villa Bianca et al. 1999). The effect of glibenclamide solution was tested in rats by measuring blood glucose 3 hours after the intraperitoneal injection.

5.2.4 Imaging
We used a custom made near infrared imaging system comprised of an infrared (IR) laser and camera (see Fig 2.2). The laser used was a 250 mW, 780nm laser diode mounted in a Thorlab cage system with a 1” round 20-degree circle tophat diffuser (transmission spectrum 380 – 1100 nm; Thorlab cat# ED1-C20-MD). Lymphatic activity was visualised by injection of 50 µl of ICG-BSA (0.8mM) into the right and left side of the tail base, with fluorescence imaged using an Electron Multiplying Charge Coupled Device (EMCCD) camera (Andor, iXon Ultra 897), a near infrared filter (Thorlab, FBH850-40 - Premium Bandpass Filter, Cat. # FBH850-40) and a Fuji (DV10x7B-2) camera lens. The system was enclosed in a lightproof cage with safety cut-off switch to prevent accidental exposure. Recordings were made at a frame rate of 12.5 frames/s (exposure 0.08 s) and analysed by Image Workbench software (INDEC BioSystems, USA).

5.2.5 Cytokine measurement
Serum samples were obtained from 200 µl whole blood samples collected from the tail artery before LPS or saline treatment and 4 hours after LPS or saline treatment. The blood was deposited in K2E/K2EDTA tubes (VACUETTE® K2EDTA tubes, Greiner Bio-One, Austria) at room temperature for 30 minutes and then centrifuged at 3000g for 10 minutes at 20°C. Each serum sample was transferred into a 2 ml eppendorf tube and stored at -20°C until analysis. Samples were diluted 4 times according to manufacturer’s instruction (Bio-Rad, Bio-Plex Pro™ Rat Cytokine Group I, 23-Olex Assay, Cat. #L8001V11S5). The target cytokines included the following: interleukin-1α (IL-1α), IL-1β, IL-2, IL-4, IL-5, IL-6, IL-7, IL-10, IL-12(p70), IL-13, IL-17, IL-18, EPO, G-CSF, GM-CSF, GRO/KC, IFN-γ, M-CSF (macrophage colony-stimulating factor), MIP-3α, RANTES (Regulated on Activation, Normal T Cell Expressed and
Secreted), TNF-α, VEGF and MCP-1. Cytokine/Chemokine levels were measured with a Bio-Plex® 100 System (Bio-Rad, USA).

5.2.6 Analysis

Lymphatic contraction frequency was obtained by selecting two regions of interest (ROIs) along the lymphatic vessel, with fluorescence intensity of these ROIs measured from 10-minute video recordings using Imaging Workbench. The fluorescence intensity values were plotted against time in AxoGraph (AxoGraph Scientific Sydney, Australia), and the positive and negative peaks counted on the plots, and averaged per minute (Fig. 5.2A and B).

The lymphatic contraction wave velocity was measured by in-house Matlab-integrated software. Firstly, a one pixel wide free-hand line selection was made over the lymphatic vessel segment (Fig. 5.2C) with a spatio-temporal map then generated for 5-minute images. The contraction wave demonstrated as the higher pixel line (Fig. 5.2D) was used to obtain the length (L) and time (t), with the velocity calculated as $V = \frac{L}{t}$ and the average velocity calculated from contraction waves within 5 minutes.
Figure 5.2: Contraction frequency and contraction wave velocity measurement

(A) Two regions of interest (ROIs) selected for detecting the near infrared (NIR) signal. (B) A plot of the NIR signal for an ROI. Contraction frequency was measured by counting the peaks using two ROIs (here two peaks shown with arrows). Noise produced by respiratory movement was easily distinguished by its relatively high frequency and low amplitude. (C) Line used for producing the spatio-temporal map in D (direction is from foot to head). (D) spatio-temporal map: Lymph contraction wave velocity (V) and 5-minute contraction wave travel distance (L₅) were calculated by measuring the summed distance that individual contraction waves travel when measured over a 5 minute period on the same vessel segment. Here V = L/t (V-velocity, L-length, t-time and L₅ = a + b…. where the lower-case letters represent the length that individual contraction waves travel).
We also calculated the 5-minute contraction wave travel distance by adding up all individual travel distances of contraction waves within 5 minutes ($L_5$) over the same vessel segment, calculated by the following: $L_5 = a+b+...$. Where $a$, $b$,... represent the distances contraction waves travelled with each contraction (Fig. 5.2D).

### 5.2.7 Statistical analysis

Contraction frequencies, contraction wave velocity and 5-minute lymph travel distances were compared for statistical significance using two-way ANOVA in GraphPad Prism 6. Values are expressed as mean ± SD with $P < 0.05$ considered significant and $n$ referring to the number of animals used.

### 5.3 Results

#### 5.3.1 Near infrared (NIR) imaging of rat inguinal-to-axillary lymphatic vessels

Video images recorded from inguinal-to-axillary lymphatic vessels showed active propulsion of lymph (contractions seen as peaks, Fig. 5.2 A and B). Two (i.e. ventral and dorsal) pathways of lymphatic vessels from the injection site to the inguinal lymph node were found to be present in most animals, in which case video recordings were made from lymphatics in both pathways. The ventral pathway (Fig. 5.3 arrow 1) has lymphatic vessels that traverse from the base of the tail along the front of the inner side of the groin to above the anterior superior iliac region near the spine where they meet the inguinal lymph node. The dorsal pathway (Fig. 5.3 arrow 2) has lymphatic vessels that primarily traverse from the base of the tail past the anterior superior iliac region near the spine to the inguinal lymph node. Lymph flow then continues through lymphatic vessels that emerge from the inguinal node and continue to the axillary node (Fig. 5.3).
Figure 5.3: Left side lymphatic vessels and inguinal lymph node

(A) Near infrared image of left side lymphatic vessels and inguinal lymph node (located at the intersection of ventral and dorsal pathways) observed after injection of 50 µl ICG-BSA solution at the base of the tail. (NB. both sides of lymphatic vessels were visualised in this study, here only the left side is demonstrated). (B) Schematic illustration of lymphatic vessels on 3D Rat model. Arrow 1: ventral pathway, arrow 2: dorsal pathway, arrow 3: inguinal lymph node and inguinal-to-axillary lymphatic vessel.
Measurement of lymphatic activity was made using NIR imaging of ICG. Measurements under control conditions provided a mean contraction frequency of the inguinal-to-axillary lymphatics of $13.4 \pm 2.1$ contractions/min (right) and $11.8 \pm 2.4$ contractions/min (left) ($n=21$ rats). The lymphatic contraction frequency and contraction wave velocity were not significantly different for the two sides, the average of the two being $12.6 \pm 2.4$ contractions/min and $1.0 \pm 0.2$ cm/s. Surprisingly, lymphatic contractions varied substantially depending on the region. For example, the average frequency for the inguinal-to-axillary lymphatics was approximately 50% higher than the average frequency of $8.3 \pm 2.2$ contractions/min ($n=5$) for groin lymphatic vessels in untreated (i.e. control) rats ($P < 0.0001$) and the average contraction wave velocity of the inguinal-to-axillary lymphatics was almost double the average velocity of $0.5 \pm 0.1$ cm/s ($n=5$) recorded from the groin lymphatics ($P < 0.0001$).
Figure 5.4: Comparison of cytokine levels after 4 hours of LPS administration
IL-6, MCP-1, GRO/KC but not IL-1β levels increased significantly after 4 h LPS administration (n=5, all values compared to corresponding pre-treatment values, Glibenclamide-G, Vehicle-V).
* $P < 0.05$, *** $P < 0.01$. 
5.3.2 Significant inflammation induced by LPS administration

Cytokine/chemokines (23 in all, Methods) were measured before and after 4 hours of LPS with the study made using four groups, namely: saline (control), LPS, LPS-glibenclamide (treated with glibenclamide after 3 hours of LPS administration) and LPS-vehicle groups (treated with vehicle after 3 hours of LPS administration). IL-6, MCP-1 and GRO/KC were significantly elevated in the LPS group (IL-6 $P < 0.05$, MCP-1 and GRO/KC $P < 0.01$, see Fig. 5.4). High levels of MCP-1 and GRO/KC were also observed in LPS-glibenclamide and LPS-vehicle groups ($P < 0.01$, Fig. 5.4). TNF-$\alpha$ and M-CSF concentrations were higher in the LPS-vehicle group compared to in the saline group (TNF-$\alpha$: 422 ± 280 pg/ml in saline, 1167 ± 543 pg/ml in LPS-V. M-CSF: 901 ± 439 pg/ml in saline, 1553 ± 175 pg/ml in LPS-V, both $P < 0.05$). IL-1$\alpha$, IL-1$\beta$, IL-2, IL-4, IL-5, IL-7, IL-10, IL-12 (p70), IL-13, IL-17, IL-18, EPO, GM-CSF, IFN-$\gamma$, M-CSF, MIP-3$\alpha$, RANTES and VEGF concentrations were not elevated in any groups (n=5 for each group).
Figure 5.5: Effects of LPS on contraction frequency and wave velocity

Contraction frequency and wave velocity measured from 5-minute near infrared (NIR) video recordings for both right and left inguinal-to-axillary rat lymphatic vessels. Both contraction frequency and wave velocity on right and left side did not alter significantly (n=5, all values compared to corresponding baseline pre-treatment values, glibenclamide-G, vehicle-V).

5.3.3 Low-moderate levels of local inflammation do not alter lymphatic contractile properties

We measured contraction frequency, contraction wave velocity and 5-minute lymph travel distance in order to assess lymphatic function before or after LPS treatment. The basal contraction frequency for right and left sides was 13.5 ± 2.6 contractions/min and 13.6 ± 2.7 contractions/min respectively (Fig. 5.5A and 5.5B, LPS group, n=5). There was no significant change even after 4 hours of LPS administration (right 9.9 ± 1.9 contractions/min, left 11.1 ± 2.9 contractions/min, P > 0.05), when compared to either its basal value or control value. The contraction wave velocity before treatment was 0.8 ± 0.1 cm/s (right) and 0.9 ± 0.2 cm/s (left) and these parameters were also not altered by LPS administration (4 h post-LPS, right 0.8 ± 0.2 cm/s, left 0.9 ± 0.3 cm/s, P > 0.05). Glibenclamide did not significantly alter the above parameters (Fig. 5.5C and 5.5D) or
cytokine levels in LPS-induced inflammation (Fig. 5.4). However, the 5-minute lymph travel distance was reduced after 4 hours of LPS administration for both the right (from 120 ± 34 cm to 58 ± 11 cm, $P < 0.01$) and left sides (from 130 ± 96 cm to 75 ± 39 cm, $P < 0.05$) in the saline-treated group but not in the LPS group (Fig. 5.6).

**Figure 5.6: Effects of LPS on lymphatic flow**

Lymphatic flow, measured as the 5-minute travel distance was reduced in the left (A) and right (B) side lymphatic vessels after 4 hours of saline administration (left $P < 0.05$, right $P < 0.01$) but was not significantly altered in the LPS treated animals. Glibenclamide did not have a significant effect. (glibenclamide-G, vehicle-V)

### 5.4 Discussion

This study, made using near infrared imaging of Indocyanine Green to visualise the activity of rat inguinal-to-axillary lymphatic vessels, demonstrated two pathways for lymph flow between the tail injection site and the inguinal lymph node. This differs to the finding in mice where one pathway was demonstrated. A previous investigation in rat did not show these two pathways (Suami, Chang et al. 2011).

#### 5.4.1 Regional differences in lymphatic contractile properties

Inguinal-to-axillary lymphatic vessels exhibited a 50% higher contraction frequency compared to lymphatic vessels from the groin region for baseline values. The contraction wave velocity was also faster being double that of groin lymphatic vessels. These findings suggest regional differences in lymphatic contractile properties. Gashev
and his team also reported lymphatic regional differences in rat isolated thoracic duct, neck, mesentery and hind limb in response to various perfusion pressures (Gashev, Davis et al. 2004).

5.4.2 LPS-induced inflammation and lymphatic function

LPS produces dose- and time-dependent increases in cytokine levels (Frost, Nystrom et al. 2002). In our study, local foot injection of LPS at 1.65 mg/kg induced significant inflammation and significantly increased levels of IL-6, MCP-1 and GRO/KC. However IL-1β, one of the key cytokines, was not measurably elevated. This may relate to the peak level of IL-1β having already returned to baseline levels, as our blood samples were taken 4 hours after LPS administration whereas peak IL-1β levels are reported to occur 2 hours after i.p. LPS administration (Nezic, Skrbic et al. 2009). The time at which peak cytokine levels occur may also vary with the dose of LPS and the route of administration. Therefore it is not possible to detect all peak values by measurement at one time point (Roth, McClellan et al. 1994, Tateda, Matsumoto et al. 1996).

The level of MCP-1 in this study was comparable to that measured in similar mice model with a double dose of LPS administration (Aldrich and Sevick-Muraca 2013). But IL-6 was elevated only half the level. The 1.65 mg/kg LPS dose of our studies caused significant systemic inflammation. However, the lymphatic system was remarkably resilient to the inflammation with the contraction frequency and contraction wave velocity not significantly altered. In contrast, the effects of LPS challenge on mice at a much higher dose (100µg/mouse, 3.3 ~5 mg/kg estimated on average 20 ~ 30 g body weight) inhibited lymphatic pumping (Aldrich and Sevick-Muraca 2013). LPS directly upregulates cytokine production in lymphatic endothelial cells (LEC) by activation of NF-κB, and c-Jun N-terminal kinase, and results in increase of IL-6 and IL-8 (Sawa, Ueki et al. 2008). LPS also stimulates macrophages to release IL-1 (Dinarello 1996), IL-6 (Akira, Taga et al. 1993), TNF-α (Tracey and Cerami 1994) and many other cytokines. Cytokines such as IL-1β and TNF-α have an anti-lymphangiogenic effect at higher concentrations (Chaitanya, Franks et al. 2010) suggesting cytokines may also disrupt this lymphatic property in a dose dependent manner. In the case of our study the increased levels of cytokines IL-6, MCP-1 and GRO/KC were not sufficient to impede lymphatic function.
A notable observation was that the 5-minute lymph travel distance was significantly reduced in saline-treated but not in the LPS groups suggesting that LPS-induced inflammation enhanced lymphatic function. The decreased 5-minute lymph travel distance under control conditions could relate to impairment by ICG itself (Gashev, Nagai et al. 2010). However this should be the same for all conditions and hence the finding that lymphatic function was enhanced by LPS remains valid. A likely explanation is that the propulsion of lymph is augmented with inflammation by increased interstitial tissue pressure, as LPS injection generally caused oedema local to the site of injection. The result is consistent with the observation of increased lymphatic stroke volume without alteration of contraction frequency in acute inflammation (Benoit and Zawieja 1992). A greater stroke volume may enable lymph to travel further through contraction of lymphatic chambers (i.e. lymphangions) under higher luminal pressure.

5.4.3 LPS, NO and lymphatic function
We did not measure NO production in our study, but it is worthy of discussion as it has a close link with lymphatic contractile properties. NO can inhibit lymphatic pumping by reducing the frequency and amplitude of contractile activity (Gasheva, Zawieja et al. 2006), though its effects in vivo may be more complicated (Liao, Cheng et al. 2011). LPS produces a dose-dependent increase in nitrite production in cultured murine macrophages RAW 264.7 (Kiemer, Muller et al. 2002, Aldridge, Razzak et al. 2008) and rat mesenteric lymphatic vessel smooth muscle cells (Robertson, Hughes et al. 2004). Presumably levels of NO produced by the LPS dose we used were below that which alters lymphatic function.

In LPS-induced septic shock, LPS leads to higher levels of NO production and subsequent fall in blood pressure. Glibenclamide can inhibit generation of NO, and inhibits the hypotension if used in the early stage (i.e. within 1 hour of LPS administration) (Wu, Thiemermann et al. 1995). LPS also upregulates K$_{ATP}$ channels and increases their activity in vascular smooth muscle (Shi, Cui et al. 2010). Therefore, vascular smooth muscle is relaxed by both upregulation of K$_{ATP}$ channels and NO overproduction. The same may occur in lymphatic smooth muscle. The fact that the 1.65 mg/kg dose of LPS did not alter the contraction frequency and wave velocity indicates that these factors were not significantly activated. Glibenclamide has anti-inflammatory properties by reducing overproduction of pro-inflammatory cytokines.
(Schmid, Svoboda et al. 2011). In our study, glibenclamide did not decrease the cytokine levels and did not alter lymphatic function suggesting that, for the conditions of our study simulating the onset of infection, $K_{\text{ATP}}$ channels were not involved in the regulation of cytokine production.

### 5.5 Conclusions

Contrary to our hypothesis, we found that moderate levels of LPS-induced inflammation did not alter lymphatic contraction frequency and wave velocity. However, overall lymph movement, as assessed by the summed distance the waves of contraction moved in a 5-minute period was increased compared to saline control. This may be consistent with increased oedema caused by LPS. The $K_{\text{ATP}}$ channel blocker, glibenclamide, did not reduce cytokine production when administered 1 hour after LPS treatment.
Chapter 6. In Vivo Investigations into the Effects of Nifedipine on Rat Lymphatic Function

6.1 Introduction

It has been reported that calcium channel blockers (CCBs) such as nifedipine and amlodipine cause peripheral oedema. The Goal in Hypertension Treatment study (INSIGHT) reported that 28% of nifedipine treated hypertension patients suffer from peripheral oedema, 8.4% of them withdrawing due to this adverse effect in the period of a 3-year study (Brown, Palmer et al. 2000). The second Swedish Trial in the Old Patients with Hypertension study (STOP-2) observed that 25.5% of patients who took either felodipine or isradipine had oedema (Hansson, Lindholm et al. 1999). Nifedipine sustained release tablets increase oedema in a dose-dependent manner (i.e. oedema increased by 8%, 12% and 19% corresponding to 30 mg, 60 mg and 90 mg nifedipine tablets respectively) (Bayer 2009). This problem needs to be resolved, as CCB-induced peripheral oedema causes withdrawal of the drug treatment due to this side effect (Brown, Palmer et al. 2000) or leads to the use of co-drug treatments including diuretic, venodilators, angiotensin converting enzyme (ACE) inhibitors, angiotensin receptor blockers (ARBs) or nitrates to help reduce the oedema (Pang 2001).

Oedema is seen in many diseases, such as heart failure, nephrotic syndrome, hypoproteinaemia, cirrhosis, pregnancy, chronic venous insufficiency, lymphedema, myxoedema and it occurs more readily in the legs and feet due to hydrostatic forces, especially in the elderly and/or at the end of the day after prolonged standing (Vacek 2000, Cho and Atwood 2002). In the case of CCBs such as nifedipine, oedema has been proposed to occur due to increased permeability of postcapillary venules (Taherzadeh, Das et al. 1998) and increased intra-capillary hydrostatic pressure (Valentin, Ribstein et al. 1989). It is also possible that nifedipine compromises lymphatic function and that this exacerbates oedema. However, while nifedipine inhibits the amplitude and frequency of contractions in isolated lymphatic vessels in a concentration-dependent manner (Lee, Roizes et al. 2014), a recent study by Telinius et al. (Telinius, Mohanakumar et al. 2014) that used ‘Near Infrared Imaging’ (NIR) of ICG did not reveal an effect of nifedipine on lymphatic contractile activity in healthy human
subjects, despite finding significant inhibition by nifedipine on isolated human lymphatic vessels.

The contradictory *in vivo* compared to *in vitro* findings in this human lymphatic study is a surprising outcome and worthy of further investigation. We tested the hypothesis that *calcium channel blockade reduces in vivo lymphatic contractile activity* in rats by measuring the effects of systemic application of nifedipine, at a concentration sufficient to significantly reduce blood pressure, on lymphatic contractile function.

### 6.2 Methods

#### 6.2.1 Experimental Protocol

All animal protocols and procedures were approved by The University of Newcastle Animal Care and Ethics Committee (approval: A-2009-153). Wistar outbred rats (weight 317 ± 40 g, age 8-10 weeks) were bred by the Animal Services Unit at the University of Newcastle. Rats were anesthetized with isoflurane after which the hair on the lower abdomen was shaved with a pet clipper and covered with hair-removal cream (Nair Dry & Sensitive, Church & Dwight, Sydney) for 5 min. The cream and loose hair was gently removed with a plastic scraper and the remaining cream rinsed off with warm water (post-hair removal skin shown in Fig. 6.1A).

On the second or third day after shaving, rats were deeply anesthetised by i.p. injection of urethane (1-1.5 g/kg at 0.25 g/ml in PBS solution) and placed on a heating pad to maintain rat body temperature at 37°C monitored by a rectal thermocouple. A cannula was then implanted in the carotid artery to measure rat blood pressure (see below), which together with body temperature and blood oxygen saturation were recorded (Labchart 7, AD Instruments, Australia). Rats were stabilised for 1 h at which time indocyanine green (ICG) combined with bovine serum albumin (BSA) was injected intradermally (0.8mM, volume 50 µL per injection site) with a 31-gauge needle (Becton Dickson Pty Ltd, USA) into the left and right groin regions adjacent to the base of the rat-tail (Fig. 6.1B, sample image showing intradermal injection). The ICG was imaged by exciting its fluorescence using an infrared laser before and during intravenous infusion (Harvard Infusion Pump) of nifedipine into a jugular vein at rates of 200 µg/kg/min or 400 µg/kg/min for 15 min. The total volume of nifedipine solution was 3
ml for each intravenous infusion. Upon completion of the experiment the rat was euthanized.

Figure 6.1: Procedure for near infrared (NIR) imaging of ICG from rat lymphatic vessels

*in vivo*

(A) Shaved rat with depilatory cream applied to remove the residual hair. Black dotted lines represent the approximate location of groin lymphatic vessels with black arrows showing the direction of lymph flow. (B) Sample image showing an indocyanine green-bovine serum albumin (ICG) intradermal injection on the left side hindquarter adjacent to the base of the tail. (C) NIR lymphatic image of the left and right groin lymphatic vessels. The white arrows show the direction of lymph flow for both left and right vessels.
Fig. 6.2 presents a schematic illustrating the experimental protocol. Following ICG injection and equilibration as described above, video recording of the left and right groin lymphatic vessels was commenced. After 15 min of recording, nifedipine was infused intravenously at 200 μg/kg/min for 15 min with video recording made for the last 10 min of this infusion. As the nifedipine infusion caused a reduction in blood pressure, recording was stopped for 40-60 min until blood pressure had returned to baseline after which another 15 min of recording was performed. At this point another 30 μl of ICG was injected at the same location and a subsequent 15 min video recording of groin lymphatic activity was made before commencement of nifedipine infusion at 400 μg/kg. Lymphatic activity was recorded for the last 10 min of this infusion. Blood pressure returned to baseline levels after ~ 2 hours when a further 30 μl of ICG was injected followed by 15 mins of video recording. Videos of 10 min duration taken at each time point were used for analysis. Video recordings were discarded if there was insufficient NIR signal.

Figure 6.2: Experimental design
The upper timeline represents the near infrared (NIR) imaging recording with the actual recording period in green. The lower timeline represents nifedipine infusion and the actual infusion period in red.
6.2.2 Arterial and venous cannulation
The left carotid artery and jugular vein cannulation were the first procedures performed once the rat had become deeply anaesthetised (see above). Arterial cannulation involved carefully exposing the left carotid artery and then ligating the distal end with cotton thread and clamping the proximal end. A small incision (v-shaped cut, 45º) was made with the spring-scissors on the upper surface of the artery. A 15 cm long polyethylene tube (I.D 0.58 mm, O.D 0.96 mm, Dural Plastics & Engineering, Cat# 112031) connected to 10 ml syringe (Terumo) filled with 50 IU/ml heparin was inserted, and pushed slowly ~2 cm towards the main artery with the proximal end then ligated with cotton thread to fix the cannula. The jugular vein was cannulated in the same way. The jugular vein line was connected to a 10 ml syringe filled with saline. All tubes were flushed with 1 ml saline or 50 IU/ml heparin after cannulation. Blood pressure was continuously recorded on a PowerLab 4/30 system (AD Instruments, Australia).

6.2.3 Reagents
6.2.3.1 Indocyanine green-bovine serum albumin solution: ICG and BSA were purchased from Sigma-Aldrich (ICG Cat# I2633, BSA Cat# A4503). ICG was made up to a final concentration of 0.8 mM in PBS containing 53 mg/ml BSA. The ICG-BSA solution was stored in the dark and used within 8h of preparation.

6.2.3.2 Nifedipine solution: A stock solution of 20 mg/ml nifedipine (Sigma, Cat# N7634) was made up in 40% w/w polyethylene glycol in distilled water. This stock was further diluted with saline to the final concentration for infusion.

6.2.4 NIR lymphatic Imaging
We used a custom made near infrared lymphatic imaging system, comprised of an infrared (IR) laser and camera. The laser used was a 250mW, 780nm laser diode mounted in a Thorlab cage system with a 1" round 20-degree circle tophat diffuser (transmission spectrum 380 – 1100 nm; Thorlab cat# ED1-C20-MD). ICG fluorescence was captured with an Electron Multiplying Charge Coupled Device (EMCCD) camera (Andor, iXon Ultra 897), with near infrared filter (Thorlab, FBH850-40 - Premium Bandpass Filter, Cat# FBH850-40) and a Fuji (DV10x7B-2) camera lens. The whole system was enclosed in a lightproof cage with safety cut-off switch to prevent accidental exposure. The video was recorded at a frame rate of 12.5 frames/s and analysed by Image Workbench software (INDEC BioSystems, USA).
6.2.5 Analysis

The contraction wave velocity was calculated with in-house Matlab-integrated software. Briefly, one pixel wide freehand ROIs were drawn (See Fig. 2.8A). Time series traces for fluorescence intensity were generated for the chosen time period and a spatio-temporal map generated (See Fig. 2.8B). From this spatio-temporal map contraction wave velocity was calculated by marking two points (X1, Y1) and (X2, Y2) where X represents spatial pixel location and Y time in seconds. Velocity was then calculated as 

\[ V = \frac{L}{t} \text{ (pixels/s)} \]

with \( L = Y_2 - Y_1 \text{ (pixels)} \) and \( T = X_2 - X_1 \text{ (s)} \) where \( V \) = velocity, \( L \) = length (pixels) of the measured lymphatic vessel and \( T \) = time (s). Final velocity in cm/s was calculated by calibrating the inter pixel distance in cm using a graticule.

Contraction frequency of lymphatic vessels was measured; see Chapter 2 section 2.2.5.2 and result section.

6.2.6 Statistical analysis

Data were analysed using MS Excel 2013 or Prism 6 (Graphpad, USA). Analysis involved performing a one-way repeated measures ANOVA with Bonferroni multiple comparisons posthoc test (Prism 6. All values are expressed as Mean ± SD with \( n \) referring to the number of animals used).
6.3 Results

6.3.1 NIR image collection and lymphatic flow and contraction properties

Bright fluorescence was observed in the groin lymphatics near the ICG injection site in both groins (Fig. 6.1C). Background fluorescence tended to be stable exhibiting a similar level for the first 15-30 min.

![Image of lymphatic contraction](image)

**Figure 6.3: Pattern of lymphatic contraction.**

Images A-D showing forward (LHS) or backward (RHS) sequential lymphatic contractions. The arrows show sequential positions where there is prominent forward flow of lymph fluid (i.e. from the distal to proximal end). In the backward contraction, the arrows show sequential positions where there is sequential vessel constriction rather than lymph flow. The backward contractions do not produce lymph flow due to valves in between lymphangions that prevent backflow.
Peripheral to central lymphatic vessel contractions (i.e. forward contractions) were most commonly observed, each causing forward propulsion of lymph, though in some cases retrograde contractions (backward contraction) were also observed (Fig. 6.3). Contraction wave velocity was determined from the vessels that exhibited forward contractions by producing a space-time plot (see Fig. 2.8B). Contraction wave velocity was $0.48 \pm 0.14 \text{ cm/s}$ and $0.58 \pm 0.06 \text{ cm/s}$ in the left and right groin lymphatic vessels respectively ($n=5$).

Lymphatic contraction frequency was calculated by quantifying changes in the fluorescence intensity of an appropriately placed region of interest (ROI) (see Fig. 2.7). Fluorescence intensity changes in a ROI corresponded to three different phases of lymphatic contraction: 1) a diastolic phase with intermediate diameter, which is a phase with no contraction activity between contraction cycles; 2) dilated phase corresponding to invasion of lymph fluid from upstream neighbour lymphangions to cause a dilation of a lymphangion; 3) a systolic (i.e. constricted) phase, which can occur after either the dilated or diastolic phase (See Fig. 2.7). The contraction cycle generated by a dilation-systole-diastole pattern produces a positive peak in fluorescence plot, whereas a contraction cycle generated by a systole-dilation-diastole pattern produces a negative peak. It was also possible to discriminate forward and backward contractions, which were seen as positive and negative peaks respectively. In some cases we observed contractions arising in the middle of a lymphatic chamber and propagating in both forward and backward directions suggesting a pacemaker region. Contraction frequency, calculated by counting fluorescence peaks for 10 min intervals, provided values of $7.8 \pm 1.9$ and $8.8 \pm 2.6$ contractions/min for the left and right groin lymphatic vessels (Fig. 6.4, $n=5$ for each).

6.3.2 *Nifedipine does not inhibit the contraction frequency and wave velocity*

The above baseline contraction frequencies of left and right groin lymphatic vessels were not significantly altered during 200 or 400 $\mu$g/kg/min nifedipine infusion with contraction frequency remaining effectively unchanged through the entire 4 h recording period (Fig. 6.4). Contraction wave velocity was also measured before, during and after 200 or 400 $\mu$g/kg/min nifedipine infusion, with mean left and right groin lymphatic flow velocities of $0.48 \pm 0.14 \text{ cm/s}$ and $0.58 \pm 0.06$ also not significantly altered (Fig. 6.4). In contrast nifedipine reduced the mean arterial blood pressure (MAP) measured 1 min
after nifedipine infusion by 13.5% (from 112 ± 8 mmHg to 96.8 ± 8.8 mmHg) at 200 µg/kg/min infusion rate (P<0.01, repeated measures one way ANOVA), and by 20.1% (from 112 ± 8 mmHg to 89.4 ± 8.8 mmHg; P<0.01) at 400 µg/kg/min infusion rate (Fig. 6.7). MAP returned to baseline levels (111 ± 8 mmHg) 40-60 mins after 200 µg/kg/min and (110 ± 8 mmHg) 120~150 mins after 400 µg/kg/min nifedipine infusion was stopped. The initial heart rate of 424 ± 63 beats/min was not significantly altered by nifedipine at either infusion rate (Fig. 6.5).

![Figure 6.4: Lymphatic contraction frequency and wave velocity](image)

Left and right groin lymphatic vessel contraction frequency and wave velocity pre-treatment, during treatment with a nifedipine dose rate of 200 µg/kg/min and 400 µg/kg/min and post-treatment (n=5 for each).
Figure 6.5: Effect of nifedipine on arterial blood pressure (ABP) and heart rate (HR)

Sample ABP recordings taken before and upon IV administration of two different dose rates of nifedipine (A: 200 µg/kg/min, B: 400 µg/kg/min). The upper edge of each recording is the systolic blood pressure (SBP), the lower edge is the diastolic blood pressure (DBP) and the white band in the middle is the mean blood pressure (MBP). The red line indicates the starting point for nifedipine infusion. The lower panel presents summary data (C) for the MBP and (D) for heart rate (beats/min). The first recovery measurement was taken at 40-60-min post-infusion of nifedipine at 200 µg/kg/min and the second recovery measurement was taken 2-hour post-infusion of nifedipine at 400 µg/kg/min (n=5 for each).
6.4 Discussion

Rats have been widely used as a model for a number of cardiovascular and related disorders including hypertension, stroke, diabetes, and sepsis. Our aim in this study was to develop a NIR model of lymphatic function and then use this to test the effects of a commonly prescribed calcium channel antagonist on lymphatic function.

This study further confirms the use of NIR lymphatic imaging as a viable non-invasive way of assessing lymphatic function without damaging lymphatic tissue. NIR imaging in this model (groin lymphatic imaging model) demonstrated a number of advantages: 1) left and right groin lymphatic vessels are visualized and recorded simultaneously; 2) visualization quality in the groin region is greater than in other regions such as inguinal or leg lymphatics as the groin skin is thinner allowing better illumination and emission from the dye.

Image analysis of ICG fluorescence localised to groin lymphatic vessels revealed contraction frequencies and wave velocities of ~ 8/min and 0.6 cm/s. These values were not dependent on the side of the groin on which they were recorded and are similar to values reported by previous in vivo studies of mice (Aldrich, Davies-Venn et al. 2012). The rat contraction frequency ~ 8 contractions/min is much higher than the value of 1.2 ± 0.1 contractions/min in human leg (Telinius, Mohanakumar et al. 2014). However, the contraction frequency (about 40 contractions/min) obtained from mice leg lymphatic vessels with intravital imaging is far faster than any values known so far (Liao, Cheng et al. 2011). This is because high sensitivity of this method that could detect smaller contractions. In contrast, contraction wave velocity is faster in human subjects compared to rat (human 1.2 cm/s (Telinius, Mohanakumar et al. 2014), Rat groin lymphatic 0.5-0.7 cm/s).

The role of L-type calcium channels in smooth muscle contraction is widely known (Jaggar, Wellman et al. 1998, Davis and Hill 1999, Moosmang, Schulla et al. 2003) and studies of isolated lymphatic vessels show that calcium channel antagonists can inhibit contraction (Lee, Roizes et al. 2014, Telinius, Mohanakumar et al. 2014). For example, nifedipine (10^-7 - 10^-6 M) inhibited fluid propulsion in bovine mesenteric lymphatic vessels in vitro at 4 cmH2O and 6 cmH2O transmural pressure (Atchison and Johnston 1997). Importantly, nifedipine at a lower concentration (300 nM) reduced the amplitude
of contractions in rat mesenteric lymphatic vessels \textit{in vitro} subjected to transmural pressures between 0 - 12 cmH$_2$O but did not alter contraction frequency indicating that nifedipine does not challenge the underlying pacemaker mechanism (Lee, Roizes et al. 2014). Nifedipine at concentration of 500 nM or greater is reported to completely block rat mesenteric lymphatic contractions \textit{in vitro} (Lee, Roizes et al. 2014). In human, as opposed to rat, the concentration of nifedipine to completely inhibit the contractile activity \textit{in vitro} is considerably lower (10-100 nM) (Telinius, Mohanakumar et al. 2014).

Together these \textit{in vitro} data suggest that the lymphatics could be a potential pathway for nifedipine-induced oedema, as lymphatic function is closely linked to interstitial fluid removal and impairment could exacerbate oedema. However despite this presumption, there was no evidence of impairment of rat groin lymphatic function \textit{in vivo} for nifedipine infusion at concentrations sufficient to produce a marked fall in mean arterial pressure. This result is consistent with the findings of a recent study of CCBs in human subjects where lymph flow was not significantly changed by nifedipine \textit{in vivo} for nifedipine infusion at concentrations sufficient to produce a marked fall in mean arterial pressure. In their study, they administered oral nifedipine tablets, which induced plasma concentrations that were considered to be comparable with concentrations that did reduce lymphatic function \textit{in vitro}.

Several factors may contribute to the current \textit{in vivo} results. Firstly, the real amount of nifedipine that lymphatic smooth muscle is exposed to may be less \textit{in vivo} than for the \textit{in vitro} studies. The parameter used to indicate the level of nifedipine in the body is blood plasma concentration of nifedipine. We did not directly measure this but estimated it based on a typical rat blood volume of ~20 ml for rats of weight ~320 g (Lee and Blaufox 1985) which for 15-min infusion at 400 µg/kg/min would equate to a blood concentration of ~96 µg/ml corresponding to ~140 µg/ml plasma concentration (95% protein binding) or 400 nM. However there will also be a decline in this concentration due to excretion etc., which we estimate to be 10 -15% over the 15 min infusion period (Grundy, Eliot et al. 1997), reducing the plasma concentration to ~350 nM. This nifedipine concentration is much higher than the peak value of 115 ng/ml plasma concentration proposed in the human study for 90 mg nifedipine extended release tablets (Bayer 2009). It is similar to the nifedipine concentration of ~ 300 nM required to reduce rat lymphatic contraction amplitude \textit{in vitro} but below the 500 nM
concentration that abolished contractions (Lee, Roizes et al. 2014). This suggests that lymphatics should exhibit weaker contractions though contraction frequency would be expected to remain normal (Lee, Roizes et al. 2014). Our data support the latter as contraction frequency did not significantly change. However, they do not indicate a weakening of contractions, as the propulsion velocity of lymph fluid was not significantly altered and hence lymphatic function was not significantly altered.

This finding suggests that the nifedipine concentration in the extracellular fluid surrounding lymphatic vessels is considerably lower than 350 nM. In contrast, the fact that nifedipine reduced blood pressure at our infusion levels indicates that there was a physiological action of nifedipine. This also raises the possibility that any nifedipine-induced inhibition of lymphatic function might be counteracted by greater baroreceptor-mediated sympathetic outflow subsequent to the nifedipine-induced reduction in blood pressure. This would increase lymphatic pumping, possibly at least in part compensating for any direct nifedipine effects (Wenzel, Allegranza et al. 1997). Regardless, our data and that from the human studies (Telinius, Mohanakumar et al. 2014) rules out compromised lymphatic function as a possible factor that underlies CCB-related oedema. Therefore, factors such as a nifedipine-induced increase in filtration (Taherzadeh, Das et al. 1998) remain a focus.

In overview the use of NIR imaging to examine interventions that have the potential to affect lymphatic function in vivo in an animal model represents an important approach for assessing pharmacological and physiological data from in vitro lymphatic experiments. Notably, as shown by our study and that in humans (Telinius, Mohanakumar et al. 2014), such results are not always as expected from the in vitro studies highlighting the importance of other factors. Furthermore, the fact that lymphatic vessels in animal models are likely to respond to pharmacological interventions in a similar manner to humans, as has been shown in the rat (Saul, Thomas et al. 2011), indicates that animal studies remain relevant.
6.5 Conclusions

Our study indicates that the L-type calcium channel blocker nifedipine at concentrations that significantly reduce blood pressure does not reduce lymphatic drainage in the early stage of the drug treatment. This is contrary to our hypothesis and suggests that increased filtration leading to excess interstitial fluid may be the major cause for nifedipine-related peripheral oedema. The long-term effect of L-type calcium channel blockers on the lymphatic system need to be further studied. NIR lymphatic imaging is non-invasive, and suitable to test the effect of many potential drugs on lymphatic function.
Chapter 7. General Discussion

The lymphatic system plays an important role in fluid homeostasis and immune cell delivery. Any disturbances to lymphatic function, including spontaneous pumping activity, may give rise to pathological changes. A number of common pathological conditions, or their treatments, have implications for lymphatic function. These include cardiovascular disease, diabetes, and inflammatory disorders. In this thesis, I investigated lymphatic function in response to three challenges: 1) The streptozotocin model of type 1 diabetes; 2) acute inflammation mimicked by intradermal injection of lipopolysaccharides (LPS); and 3) Ca\(^{2+}\) channel blockers. I first developed and optimised a near infrared imaging system to quantify lymphatic function and compared this to the well-described “ink injection” method (Saul, Thomas et al. 2011, van Helden, Thomas et al. 2014) where ink is injected into the foot, and the time measured from injection to observation in a deep groin lymphatic vessel (Chapter 3). We first looked at near infrared (NIR) lymphatic imaging obtained by exposing deep groin lymphatics to direct NIR light and emission. Finally, the imaging system was enhanced using an Electron Multiplying CCD (EMCCD) that allowed images to be obtained through the intact skin using a low intensity laser. Thus allowing a non-invasive method of quantifying lymphatic function.

7.1 Near infrared lymphatic imaging

7.1.1 Deep groin lymphatic imaging (Chapter 3)

The deep groin lymphatic vessels lie alongside the femoral artery and vein deep within the groin. The activity of these lymphatic vessels was quantified by analysis of fluorescence ICG intensity using ImageJ. The contraction frequency and first arrival time were extracted from fluorescence intensity values over time. These parameters were found to be more objective than the ink method where observer-dependent variation exists (see below - General lymphatic contractile properties). Although the near infrared system was more reliable than ink for deep groin imaging, deep groin imaging has other limitations, in particular the need for surgery to expose the vessels. Subsequently, we further developed the imaging system to allow non-invasive imaging of surface lymphatics.
7.1.2 Non-invasive lymphatic imaging (chapter 5 and 6)
Non-invasive imaging was obtained by implementing a high sensitivity camera and reducing the filter-effect of skin by shaving with depilatory cream to create smooth, non-hair skin. The EMCCD camera provided enough sensitivity in low light conditions to scan a weaker fluorescent signal over a wider area. This enabled the laser intensity to be reduced to about 0.79 W/cm². This setup showed the great importance of selecting the right detecting camera for non-invasive imaging, which enabled us to perform experiments under better physiological conditions.

7.1.3 Use of ICG as an indicator
ICG was used as fluorescent dye in our NIR imaging experiment. Gashev has criticised the use of ICG, as in an in vitro study they found that irradiated ICG inhibited rat lymphatic function in both a dose- and diluent-dependent manner (Gashev, Nagai et al. 2010). In contrast, others have demonstrated that the concentration of ICG necessary for lymphatic visualisation in vivo does not inhibit lymphatic contractile activity in mice (Marshall, Rasmussen et al. 2010, Aldrich, Davies-Venn et al. 2012). Another NIR indicator namely the dye 800CW (LI-COR Biosciences) has been proposed as a substitute for ICG (Heuveling, Visser et al. 2012) and may represent an alternative.

7.2 Animal models
7.2.1 Streptozotocin-induced type 1 diabetic rat model (Chapter 3 and 4)
There are several diabetic animal models classified based on the mechanism by which hyperglycaemia is achieved. These models include chemical induction (high dose of streptozotocin (STZ), alloxan or multiple low dose STZ), spontaneous autoimmune (NOD mice, BB rats and LEW.1AR1/-iddm rats) and genetically-induced (AKIA mice), and virally-induced models (Coxsackie B virus, Encephalomyocarditis, and killham virus) (King 2012). In our experiments we used a single intraperitoneal injection of 65 mg/kg STZ (high dose of STZ; (Akbarzadeh, Norouzian et al. 2007). The diabetogenic effect of this dose in our experiments was 83.3% in 24 hours and 100% in 48 hours (Chapter 4). This model provided us with significant hyperglycaemia allowing us to test our hypotheses that acute hyperglycaemia affected lymphatic function and that glibenclamide altered the time course of open skin wound healing in hyperglycaemia.


7.2.2 Open rat foot skin wound model (chapter 4)

We decided to create the wound on the foot to mimic the most common complication of diabetes rather than the lower (Gál P. 2008) or upper back (Mendes, Leandro et al. 2012) as has been reported previously. The lack of complications (i.e. haemorrhage, tendon, nerve damage and infection), reproducible rate of healing and minimal pain related behaviour made this an ideal wound model for our purposes.

7.2.3 Rat acute inflammation model (chapter 5)

Rat local foot inflammation was induced by single intradermal injection of 1.65 mg/kg lipopolysaccharides (LPS) at the dorsal side of the right hind foot. In this inflammation method, LPS is relatively localised and absorption is slower compared to intraperitoneal (i.p.) injection. Nevertheless, some LPS can travel through the circulation to trigger cytokine release. In contrast to this model, intraperitoneal injection of 5 mg/kg (septic dose) not surprisingly, has large systemic effects (Iwasa, Matsuzaki et al. 2014). Our aim was to investigate local inflammation without the confounding influence of homeostatic regulation in response to hypotension associated with sepsis (Wu, Thiemermann et al. 1995, Sorrentino, d'Emmanuele di Villa Bianca et al. 1999).

Local LPS produced dose- and time-dependent increases in cytokine levels (chapter 7; Frost, Nystrom et al. 2002). We found that a single intradermal injection of 1.65 mg/kg (LPS) gave rise to modest but significant levels of systemic inflammation, as demonstrated by increases in several plasma pro-inflammatory cytokines (i.e. IL-6, TNF-α), but not all cytokines.

7.3 General lymphatic contractile properties

Lymphatic pumping parameters, namely lymphatic contraction frequency and wave velocity, were obtained with NIR/ICG imaging from deep groin lymphatics, more superficial groin lymphatics and inguinal-to-axillary lymphatic vessels. Control values are presented in table 7.1 and plotted in figure 7.1.
Table 7.1: The contractile properties in different regions

<table>
<thead>
<tr>
<th>Lymphatic vessel</th>
<th>Contraction frequency (contractions/min)</th>
<th>Contraction wave velocity (cm/s)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Right</td>
<td>Left</td>
</tr>
<tr>
<td>Deep groin</td>
<td>6.4 ± 3.5</td>
<td>6.4 ± 2.7</td>
</tr>
<tr>
<td>Groin</td>
<td>8.8 ± 2.6</td>
<td>7.8 ± 1.9</td>
</tr>
<tr>
<td>Inguinal-to-axillary</td>
<td>13.4 ± 2.1</td>
<td>11.8 ± 2.4</td>
</tr>
</tbody>
</table>

Deep groin n=6, Groin n=5, Inguinal-to-axillary n=5, values are expressed as mean ± SD (see Fig. 7.1 for statistical analysis).

Figure 7.1: Contractile properties of lymphatic vessels from different regions

Both contraction frequency and contraction wave velocity in inguinal-to-axillary lymphatics were greater than in the other regions. Two-way ANOVA was used for both frequency and velocity analysis, velocity left $P < 0.0001$, right $P < 0.001$. Contraction frequency, left: deep groin vs. inguinal-to-axillary vessel $P < 0.001$, right: deep groin vs. inguinal-to-axillary vessel $P < 0.0001$, groin vs. inguinal-to-axillary vessel $P < 0.001$ (Deep groin n=6, Groin n=5, Inguinal-to-axillary n=5, values are expressed as mean ± SD.)
Inguinal-to-axillary lymphatic vessels exhibited a 50% higher contraction frequency compared to lymphatic vessels from the groin region. The contraction wave velocity was also faster being double that of groin lymphatic vessels. These findings suggest regional differences in lymphatic contractile properties. This may allow lymphatic vessels to function at appropriate rates to meet demands for propulsion of lymph in different regions of the body. Gashev and his team also reported regional differences in rat lymphatic vessels isolated from the thoracic duct, neck, mesentery and hind limb in response to various perfusion pressures (Gashev, Davis et al. 2004). Skeletal muscle contractions also extrinsically propel lymph through squeezing vessels within or possibly near to the muscle bed (Aukland and Reed 1993). The deep groin lymphatics while not within, are surrounded by skeletal muscle bundles, which may provide some external driving force to lymphatic vessels by changing the net diameter of the vessels. However, probably the most significant factor underlying the difference in contraction frequency between the two lymphatic beds is lymph load and/or stroke volume of the individual lymphangions.

We observed retrograde contraction waves in all lymphatic vessels. The occurrence of retrograde lymphatic contractions seems to be a normal property (McHale and Meharg 1992). An excessive rate of retrograde contraction may compromise lymphatic propulsion in pathological conditions. It may be important to perform lymphatic imaging either by NIR or other intravital imaging in disease models pertaining to lymphatic function to understand the significance of such retrograde contractions.

### 7.4 Diabetes and lymphatic function

Hyperglycaemia in type 1 and 2 diabetes causes deleterious effects on many organs and tissues, including the vasculature, nerves and musculature. The pathologies caused by diabetes in such organs lead to severe diseases, e.g. diabetic retinopathy, nephropathy, diabetic cardiovascular disease, gangrene and more (Gilbert 2015). The lymphatic system is also affected by hyperglycaemia which gives rise to increased lymphatic vessel density (e.g. in type 2 diabetes) (Haemmerle, Keller et al. 2013). Lymph volume has been shown to be increased in type 1 diabetic rat models (Feingold, Zsigmond et al. 1985, Moriguchi, Sannomiya et al. 2005). In our study, in the acute phase of type 1
diabetes, we found that lymphatic function (i.e. lymph arrival time and lymphatic contraction frequency) remained rather robust in spite of high blood glucose. Clearly in our model, increased glucose in the short term has no negative impact on lymphatic muscle. This suggests that long-term changes in oedema and other lymphatic-associated pathologies are secondary to glucose imbalance. This said it remains surprising that there was not an increase in contraction frequency given the expected increase in lymph formation, suggesting that glucose itself may have a small inhibitory effect on lymphatic activity.

7.5 The role of glibenclamide in diabetic wound healing

Hyperglycaemia in diabetes leads to development of many complications as mentioned above. 15% of diabetic patients suffer from foot ulcers and diabetic foot amputation. Notably, diabetic foot-associated amputations account for 84% of all lower leg amputations (Pecoraro, Reiber et al. 1990). Diabetic foot wound also brings considerably lower health related quality of life to diabetic patients due to its slow healing and recurrence (Valensi, Girod et al. 2005).

Wound healing is slowed by hyperglycaemia and other factors in type 1 and type 2 diabetes (Rosenberg 1990, Kumar, Ashe et al. 1994). This has also been demonstrated in the STZ-induced type 1 diabetic rat model (Seifter, Rettura et al. 1981, Sumi, Ishihara et al. 2014). Glibenclamide, a $K_\text{ATP}$ channel blocker, is used widely in type 2 diabetes as a hypoglycaemic drug. However, some studies reported a negative healing effect of glibenclamide in non-diabetic animal models (i.e. rabbit skin wound and rat gastric ulceration) (Toroudi, Rahgozar et al. 1999, Pipelzadeh, Pipelzadeh et al. 2003). To investigate the synergistic effect of hyperglycaemia and glibenclamide, the effects of glibenclamide was tested on induced foot wound in STZ-induced type 1 diabetic rat model (Chapter 5). It was determined that glibenclamide had a small but significant negative effect on wound healing. Importantly, glibenclamide also elevated blood glucose to levels higher than those in diabetic-glibenclamide and diabetic-vehicle groups. Therefore, a possible explanation for the worsened outcome in wound healing with glibenclamide is a differential glibenclamide-induced increase in hyperglycaemia, which while small was significantly increased compared to the diabetic-vehicle group. Thus the glibenclamide effect on wound healing in this type 1 rat model of diabetes
under the conditions of our study may simply arise through the higher glucose concentration and associated toxicity.

7.6 Inflammation and lymphatic function

Severe systemic inflammation relaxes vascular smooth muscle with a resultant reduction of blood pressure and suppression of lymphatic contractile activity (Aldrich and Sevick-Muraca 2013). The mechanisms triggered largely by lipopolysaccharides (in gram-negative bacterial infection), involve cytokines (Sawa, Ueki et al. 2008), NO (Kiemer, Muller et al. 2002, Aldridge, Razzaq et al. 2008) and other paracrine factors. While high levels of inflammation inhibited lymphatic contractile activity in a mouse lymphatic model, the effects of low to moderate levels of inflammation on lymphatic function were unknown. In our study, the modest inflammation achieved by local LPS injection into the foot improved lymphatic function after 4 hours of LPS administration, while contraction frequency and contraction wave velocity were maintained at the same level. K$_{ATP}$ channels in vascular smooth muscle are part of a relaxant mechanism in sepsis (Wu, Thiemermann et al. 1995, Sorrentino, d'Emmanuele di Villa Bianca et al. 1999) and K$_{ATP}$ channels in mesenteric lymphatic smooth muscle are also upregulated in 2,4,6-trinitrobenzenesulfonic acid (TNBS)-induced ileitis (Mathias and von der Weid 2013), suggesting the involvement of K$_{ATP}$ channels in inflammation. However the degree of inflammation varies in clinical practice. Therefore I tested the effects of a K$_{ATP}$ channel blocker (i.e. glibenclamide) locally administered to rats after inducing a low to moderate level of LPS inflammation to examine whether activity of this channel was enhanced by such inflammation. The outcome was that glibenclamide did not inhibit or improve lymphatic function after LPS inflammation, suggesting that K$_{ATP}$ channel activity in lymphatic smooth muscle is not upregulated by low to moderate LPS inflammation. Glibenclamide also did not reduce the increased cytokine level in inflammation.

7.7 Ca$^{2+}$ channel blocker associated with oedema and lymphatic function

Ca$^{2+}$ channel blockers are widely prescribed anti-hypertensive drugs. One of the adverse effects is local oedema, which leads to withdrawal of the drug treatment in some cases. The underlying mechanism of oedema (i.e. excessive fluid in the interstitial space) is
largely through leakage from the microvasculature. As lymphatic function is to remove excessive fluid, it was important to investigate the effect of Ca\(^{2+}\) channel blockers on lymphatic function during Ca\(^{2+}\) channel antagonist drug treatment. *In vitro* experiments have demonstrated that Ca\(^{2+}\) channel blockers significantly inhibit lymphatic contraction frequency and amplitude (Ohhashi, Azuma et al. 1978). After we had nearly completed our study, Telinius et al. (Telinius, Mohanakumar et al. 2014) reported the effects of nifedipine on lymphatic contractile properties by NIR in human subjects. Similar to our rat data oral nifedipine did not alter lymphatic contraction frequency and velocity of lymph flow. As for the study by Telinius et al., we were not able to measure the diameter change with NIR imaging. Therefore stroke volume was not obtained. We concluded two possible explanations; firstly, lymphatic function does not change with nifedipine treatment, and oedema results from microvasculature leakage that overwhelms lymphatic removal capacity. Secondly lymphatic output may be decreased by a smaller stroke volume. It is also possible that microvascular leakage and decreased lymphatic output may coexist.
References


References


Hagiwara, N., H. Irisawa and M. Kameyama (1988). "Contribution of two types of calcium currents to the pacemaker potentials of rabbit sino-atrial node cells."


References

References


References


References


