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

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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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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

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Abbreviations

ABP	arterial blood pressure
ACE	angiotensin converting enzyme
AIHW	Australian Institute of Health and Welfare
ARBs	angiotensin receptor blockers
BSA	bovine serum albumin
Ca^{2+}	calcium ion
CCBs	calcium channel blockers
CCD	charge-coupled device
Cl^-	chloride anion
Cl_{Ca}	Ca^{2+}-activated Cl^-channels
cm	centimeter
DM	diabetes mellitus
DMSO	dimethyl sulfoxide
EDD	end-diastolic diameter
EF	ejection fraction
EMCCD	electron multiplying charge-coupled device
ESD	end-systolic diameter
FKBP12	12-kDa tacrolimus binding protein
FREQ	contraction frequency
g	gram
GRO/ KC	growth-regulated oncogene/ keratinocyte chemoattractant
H	hypothesis
HR	heart rate
i.d.	intradermal
I.D	inside diameter
i.p.	intraperitoneal
i.v.	intravenous
ICCs	interstitial cells of Cajal
ICG	indocyanine green
If	funny current
IL-6	interleukin-6
IP_3R	inositol 1,4,5-trisphosphate receptor
K^+	potassium ion
K_{ATP}	ATP-dependent potassium channels
kg	kilogram
L	length
LEC	lymphatic endothelial cell
LPS	lipopolysaccharides
MAP	mean arterial blood pressure
MCP-1	monocyte chemoattractant protein-1
mg	milligram
ml	milliliter

MLV	mesenteric lymphatic vessels
mmHg	millimeter of mercury
mmol	millimolar
Na^+	sodium ion
nAMP	normalized contraction amplitude
NIR	near infrared
NO	nitric oxide
O.D	outside diameter
PBS	phosphate-buffered saline
PO_2	oxygen partial pressure
ROI	region of interest
s	second
SAN	sinoatrial node
SERCA	sarco/endoplasmic reticulum Ca^{2+}-ATPase
SR	sarcoplasmic reticulum
STDs	spontaneous transient depolarizations
STZ	streptozotocin
SUR	sulfonylurea receptor
t	time
T1DM	type 1 diabetes mellitus
TNF-α	tumor necrosis factor-α
V	velocity
VEGF	vascular endothelial growth factor
VIP	vasoactive intestinal peptide
µg	microgram
µl	microlitre
µm	micrometer

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 Function Remains 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

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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_{ATP} channel blocker glibenclamide did not alter inflammation, as the cytokine levels and lymphatic function were not significantly changed indicating K_{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 *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.