Towards the prevention of pressure ulcers

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SINGAPORE

TOWARDS THE PREVENTION OF PRESSURE ULCERS

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Towards the Prevention of Pressure Ulcers

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Interdisciplinary Graduate School
NTU Institute for Health Technologies (HealthTech NTU)

A thesis submitted to the Nanyang Technological University in partial fulfilment of
the requirement for the degree of
Doctor of Philosophy

2021

Statement of Originality

I hereby certify that the work embodied in this thesis is the result of original research, is free of plagiarised materials, and has not been submitted for a higher degree to any other University or Institution.

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Milton Kwek Sheng Yi

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I have reviewed the content and presentation style of this thesis and declare it is free of plagiarism and of sufficient grammatical clarity to be examined. To the best of my knowledge, the research and writing are those of the candidate except as acknowledged in the Author Attribution Statement. I confirm that the investigations were conducted in accord with the ethics policies and integrity standards of Nanyang Technological University and that the research data are presented honestly and without prejudice.

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The contributions of the co-authors are as follows:

- Prof. Becker provided the initial project direction and edited the manuscript drafts.
- I did all the experiments, analysis and provided the initial project direction and wrote some parts of the manuscript.
- Thangaveloo M, wrote the manuscript drafts and put together all the figures. The manuscript was revised by Prof. Phillips and Prof. Becker.
- Hui SLB helped with the manuscript.
- Madden LE helped in some experiments and with the manuscript.

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I am an author of Lio DCS, Chia RN, Kwek MSY, Wiraja C, Madden LE, Chang H, Khadir SMA, Wang X, Becker DL, Xu C. Temporal pressure enhanced topical drug delivery through micropore formation. Sci Adv. 2020 May 29;6(22): eaaz6919. doi: 10.1126/sciadv. aaz6919. PMID: 32523993; PMCID: PMC7259933.

My contributions are as follows:

- Teaching initial pinch model
- Writing methods and materials for figure 5.
- Performing experiments and analysis for figure 5

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Milton Kwek Sheng Yi

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Abbreviations

α SMA	α -smooth muscle actin
ADP	Adenosine diphosphate
AF	Alexa fluorophore
ANG	angiotensin
ANOVA	Analysis of Variance
asODN	DNA antisense oligonucleotide
ATP	Adenosine triphosphate
bp	Base pair
BSA	Bovine serum albumin
cDNA	Complementary deoxyribonucleic acid
CEAP	Clinical, etiological, anatomical, pathophysiological
CL	Cytoplasmic loop
Cre/lox	Cre recombinase/ LoxP site
CreER	Cre Recombinase Oestrogen receptor
CT	C-terminal tail
Ct	Cycle threshold
Cx	Connexin
DAMP	Damage associated molecular patterns
DAPI	4',6-diamidino-2-phenylindole
DEPC	Diethylpyrocarbonate
DNA	Deoxyribonucleic acid

DNase	deoxyribonuclease
DNTP	Deoxynucleotide triphosphate
E1,2	Extracellular loop 1,2
ECM	Extracellular matrix
EDTA	Ethylenediaminetetraacetic acid
EL	Extracellular loop
EMMA	European Mouse Mutant Archive
eNOS	Endothelial nitric oxide synthase
ER	Endoplasmic reticulum
EtOH	Ethanol
FA	Facility acquired
FDA	Food and drug administration
FITC	Fluorescein isothiocyanate
FRET	Förster resonance energy transfer
Gap26, 27	Connexin mimetic peptide 26,27
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GJ	Gap junction
GMCSF	Granulocyte-macrophage colony-stimulating factor
GPVI	Glycoprotein VI
HID	Hystrix-like ichthyosis with <i>deafness</i>
HMGB1	High mobility group box protein 1
HUVEC	Human Umbilical Vein Endothelial Cells
I/R	Ischaemia/Reperfusion
IACUC	The Institutional Animal Care and Use Committee

ICAMS	Intercellular Adhesion Molecule 1
IDT	Integrated DNA Technologies, Inc.
IL	Interleukin
IP3	Inositol 1,4,5-triphosphate
JNK	c-Jun N-terminal kinase
kDa	Kilo Dalton
KID	Keratitis ichthyosis deafness
KO	Knockout
LPS	Lipopolysaccharide
MCAO	Middle cerebral artery occlusion
MLKL	Mixed lineage kinase domain-like
MMP	Matrix metalloproteinases
mRNA	Messenger ribonucleic acid
NAD	Nicotinamide adenine dinucleotide
NADH	Nicotinamide adenine dinucleotide hydrogen
NADPH	Nicotinamide adenine dinucleotide phosphate
NID	Necroinflammatory debris
NO	Nitric oxide
NPUAP	National Pressure Ulcer Advisory Panel
NT	N-terminal tail
O.C.T	Optimal Cutting Temperature
ODDD	Oculodentodigital dysplasia

ODN	Oligodeoxynucleotide
OGD	oxygen glucose deprivation
P120ctn	p120ctn catenin gene
P2X	ATP-gated P2X receptors
P2Y	purinergic G protein-coupled P2Y receptor
PBS	Phosphate buffered saline
PBST	Phosphate buffered saline + Tween20
PC	panniculus carnosus
PCR	Polymerase chain reaction
PF61,127	Pluronic F61, F127
PFA	Paraformaldehyde
PU	Pressure ulcer
qPCR	Real-time polymerase chain reaction
RhoGTPase	Rho guanosine triphosphatase
RIP1,3, RIP3K	receptor-interacting protein kinase 1,3
RNA	Ribonucleic acid
RNase	Ribonuclease
RO	Retro-orbital
ROI	Region of interest
ROS	Reactive oxygen species
ROX	6-carboxy-X-rhodamine
SCC	Squamous cell carcinoma
SDS	Sodium dodecyl sulphate
ST	SenseODN treated

STZ	Streptozotocin
TE	Tris EDTA
TEK	TEK Receptor Tyrosine Kinase
TF	Tissue Factor
TK	Thymidine kinase
TM	Transmembrane
TNF α	Tumour necrosis factor alpha
TR	Treated
Tris	tris(hydroxymethyl)aminomethane
Triton-X	Polyethylene glycol p-(1,1,3,3-tetramethylbutyl)-phenyl ether
VC	Vehicle control
vWF	Von Willebrand factor
WAT	White adipose tissue
ZO-1	Zonula Occludens-1

Abstract

This study is centred around preventing pressure ulcer (PU) progression by first understanding what happens in an early experimental PU and later attempting to prevent experimental PU progression. Pressure ulcer is one of the costliest and physically debilitating condition in the 20th century. While there are many guidelines on preventing PU progression, it continues to be prevalent. This warrants for a better understanding of what causes a PU with the aim of treating the cause. Ischaemia reperfusion (I/R) injury has been one of the potential causes of PU. This injury happens as bony prominences are rested on surfaces for relatively long periods of time where tissues experience ischaemia. Tissue injury is then exacerbated by reperfusion upon pressure relief. Ischaemia reperfusion injury is well studied in ischaemic stroke and cardiac infarction situations. In both organs, connexin43 is implicated in the pathogenesis of I/R injuries and modulating connexins in these organs can reduce I/R injury. Regarding the skin, publications report the creation of an experimental PU model by inducing I/R injuries. However, few experimental PU models are characterised to study the early events of an experimental PU. To do so, an early experimental PU model is optimised and characterised. With this early experimental PU model, epidermal Cx43 expression was demonstrated to be up regulated in the skin after I/R injury at 4 h and 24 h. The up regulation of epidermal Cx43 is incidentally an observation also seen in publications regarding wound edges of human PUs. Epidermal Cx43 up-regulation was found to be prevented by treating the early PU model with Cx43 antisense oligonucleotide (Cx43asODN). Necroptosis, a cell death pathway, is also known to be involved in I/R injury. Up-regulation of both necroptosis related proteins, high mobility group box 1 protein (HMGB1) and receptor-interacting seine/threonine-protein kinase 3 (RIP3), were observed in the epidermis. Expectedly, epidermal high mobility group box 1 protein (HMGB1) and receptor-interacting seine/threonine-protein kinase 3 (RIP3) up-regulation was also inhibited when treated with Cx43asODN. Furthermore, experimental PU progression was prevented when Cx43asODN was administered in a more severe experimental PU model. These findings suggest that the treatment of Cx43asODN was successful in inhibiting I/R injury which resulted in the prevention of experimental PU progression.

Chapter 1. Introduction

1.1 The Skin

The skin is the largest organ in the body and one of its functions is to protect the body from the external environment and pathogens. The skin can be broadly separated into the epidermal, dermal and hypodermal layers [1] (Fig. 1.1).

Figure 1.1: A schematic diagram of the skin. The skin is made up out the epidermis, dermis, and hypodermis. Adapted from Elder, D.E., Lever's histopathology of the skin Eleventh edition.

1.1.1 Epidermis

The epidermis is located at the outermost layer of the skin and provides an impermeable barrier to the environment [1]. The epidermis also regulates water loss (also known as trans-epidermal water loss) [1]. Importantly, the epidermis is constantly regenerating throughout life. It is estimated that the human epidermis turns over every 40-56 days while mice have an estimated turn over rate of 8-10 days [2, 3]. Since the epidermis is

avascular, it gets its nutrients via diffusion from the vasculature of the dermis [1]. The epidermis is made up mainly of keratinocytes in progressive stages of differentiation, from the undifferentiated basal layer upwards [1]. The basal cells are cuboidal and are constantly undergoing mitosis to replace the upper more differentiated epidermal layers [1]. Cells that leave the basal layer form the stratum spinosum and express cadherins, which form desmosomes, which give the cells a spiny appearance [1]. As the keratinocytes are pushed further away from the basal layer into the stratum granulosum, they start to appear grainy and flatter. This is due to the increase in keratin and keratohyalin generation [1]. Keratinocytes in this layer undergo nuclei and organelle loss as they die [1]. This process ultimately results in a stratum corneum comprising of dead keratinocytes, which serve as a barrier against the environment [1]. The stratum basale is attached via hemidesmosomes to a highly specialized extracellular matrix (ECM) called the basal lamina (basement membrane) that provides support for the epithelia [4].

1.1.2 Dermis

The dermis, a connective tissue, is mainly populated by fibroblasts. It is located beneath the epidermis and above the subcutaneous tissue [1]. The dermis can be separated into the superficial papillary layer (upper dermis, closer to the epidermis) and the reticular layer (lower dermis that is closer to the hypodermis) [1]. Collagen fibre bundles in the papillary dermis are thin. In contrast, the reticular dermis contains thick basketweave collagen fibre bundles [1]. The dermal extracellular matrix (ECM) also contains bundles of elastin [1]. Additional structures such as hair follicles and sebaceous glands

are located in the dermis [1]. Lymphatic and blood vessels are also present in the dermis for skin metabolism and homeostasis [1].

1.1.3 Sweat and sebaceous glands

Sweat glands are also located in the dermis and have important thermoregulatory functions for the skin. The three main types of sweat glands are eccrine, apocrine and apoeccrine sweat glands [5-7]. Eccrine glands, the first to be discovered, are the most ubiquitous and are found in locations from palms and soles to hairy skin [5]. Apocrine glands are larger compared to eccrine glands and are connected to hair follicles instead of the skin surface [6]. The third type is the apoeccrine gland which shares properties of both eccrine and apocrine glands [7]. The apoeccrine gland develops from the eccrine sweat glands from the age of 8-14 years [7]. Sebaceous glands, although not a type of sweat gland, is also located in the dermis and are associated with hair follicles [8]. Sebaceous glands are holocrine glands that secrete a viscous, lipid-rich fluid called sebum [8].

1.1.4 Hypodermis

The subcutaneous layer or the hypodermis forms the last layer of the skin. The hypodermis contains larger blood vessels and mainly loose connective tissue and adipose tissue [1]. In a mouse, white adipose tissue (WAT) is situated above and beneath the panniculus carnosus (PC) while in humans adipose tissue exists under the reticular dermis [9]. The main function of WAT is to act as a form of energy store where fatty acids can be broken down into ATP when energy is needed. White adipose tissue also plays other roles such as regulating endocrine functions, glucose hemostasis, lipid metabolism, inflammation and angiogenesis [10-13]. Of late, it is emerging as a migratory tissue that plays a part in the inflammatory response to injury [14].

1.2 Pressure ulcer progression

1.2.1 Stage 1 pressure ulcer

The first stage involves closed wound damage, which is described as non-blanchable intact skin [15]. This is thought to be due to ischaemia reperfusion (I/R) injury caused by unrelieved pressure for extended periods of time and then reperfusion of blood upon the release of pressure [16, 17]. When pressure is applied to tissue overlying a bony prominence, ischaemic conditions are produced because the pressure prevents the blood flow to the area (ischaemia) [16, 17]. Cells in this state have compromised metabolic priorities. When nutrient-rich blood is restored (reperfusion), cells suffer from oxidative stress and cytotoxic injury [16, 17]. Insulted cells release damage-associated molecular patterns (DAMPs) such as high mobility group box protein B1 (HMGB1), DNA and ATP. These signals activate the production of pro-inflammatory cytokines such as interleukin-1 [18]. Damaged endothelial cells express adhesion molecules to facilitate neutrophil extravasation into the insulted area [19, 20]. Specifically, P-selectin expression peaks after 10-20 min of reperfusion on the surface of endothelial cells and are later shed in soluble fragments into the bloodstream [21-23]. Recruited neutrophils aggravate the situation by breaking down ECM with elastases and releasing reactive oxygen species (ROS), leading to more damage [24]. Neutrophils secrete more pro-inflammatory cytokines such as IL-1 α , IL-1 β , IL-6 and TNF α and chemokines attracting more neutrophils to the area [25]. Microvascular dysfunction, caused by the lack of nitrous oxide, can lead to leukocyte trapping, resulting in a “no-reflow” phenomenon [26]. Following this, the pressure ulcer may heal, or worsen into a more severe stage of PU.

1.2.2 Stage 2 pressure ulcer

When a Stage 1 PU progresses to a Stage 2 PU, the skin loses its epidermis and part of the dermis [27]. This progression into a Stage 2 PU poses infection risks as the epidermal barrier is now compromised with the loss of the epidermis. Furthermore, dermal papillae are found to be reduced at the edge of the PU and are absent in the PU area as the cells die [28]. The area is also filled with fat cells [28]. The dermis at the edge of the PUs shows thicker collagen fibres and deposits of fat [28]. Some Stage 2 PU will undergo a normal healing process but others will deteriorate.

1.2.3 Stage 3 and Stage 4 pressure ulcer

Progression to a Stage 3 PU is characterised by the transition from a partial thickness wound to a full-thickness wound that exposes underlying muscle with the presence of necrotic tissue. Finally, the ulcer progresses to Stage 4 in which the underlying muscle becomes necrotic and degrades, and the bone is exposed [29-31]. Progression from normal skin to Stage 4 PU is summarised as follows (Figure 1.2).

Figure 1.2: Cartoon of the 4 stages of PU progression in a human. a) Normal skin is allocated Stage 0. b) In a Stage 1 PU, the skin remains intact with observable non-blanchable erythema. c) In a Stage 2 PU, there is a partial thickness wound involving the loss of epidermis and dermis. d) A Stage 3 PU is a full-thickness wound exposing underlying muscle and e) a Stage 4 PU exposes the bone. Adapted from Edsberg 2007.

1.3 Wound healing

The skin goes through four overlapping stages of healing involving haemostasis, inflammation, new tissue formation and tissue remodelling [32]. Upon wounding, haemostasis is the first response. Primarily the function of haemostasis is to stop the wound from being exposed to the external environment and to stop further bleeding. Haemostasis may be further separated into primary and secondary haemostasis [33]. Primary haemostasis involves platelet aggregation forming a plug, while secondary haemostasis is the process where insoluble fibrin is produced from soluble fibrinogen to further reinforce the platelet plug [33].

Figure 1.3: Figure describing wound healing involving haemostasis, inflammation, new tissue formation and tissue remodelling. Images were taken from Kawasumi et al., 2013. a) Platelets initiate blood clotting by activating clotting factors in the haemostasis process. b) Inflammatory process, involving neutrophils and macrophages, begins immediately upon wounding that lasts for a few hours to a few days. c) New tissue formation or proliferation involving fibroblasts, endothelial cells, epithelial cells for wound closure and generation of granulation tissue. d) Remodelling of scar tissue to normal tissue in the following months.

Damaged cells release ROS resulting from NADPH-oxidase located on the plasma membrane. The ROS rapidly recruits leukocytes to the injured area [34]. Resident mast cells, resident dendritic cells and resident macrophages are also activated [35, 36]. In the first hour of injury, keratinocytes and resident macrophages produce pro-inflammatory signals [37, 38]. Resident mast cells degranulate and release histamine, leukotrienes, prostanoids and cytokines [38]. These proinflammatory signals attract neutrophils to the area to prevent infection by killing any microbes that have entered the wound. Neutrophils continue to accumulate at the injury site and macrophages start to increase in number within 2 to 3 days. Neutrophils and macrophages take over as the major producers of pro-inflammatory cytokines such as interleukin-1 α (IL-1 α), IL-1 β , IL-6, IL-12, and TNF α [39]. These cytokines are all up-regulated during inflammation to prevent infection and stimulate cellular recruitment.

Re-epithelisation occurs as keratinocytes are activated, changing their cytoskeleton and cell surface receptors. The expression of K6 and K16 allows keratinocytes to start migration [40]. Keratinocytes at the wound edge remove desmosomes and hemidesmosomes to allow migration while keratinocytes behind them proliferate [41, 42]. Keratinocytes also produce matrix metalloproteinase (MMP)-1, MMP-9, MMP-10, MMP-13, which allows them to cut a path forwards [43-45].

The formation of granulation tissue involves fibroblast and endothelial cells. Fibroblasts mainly produce and deposit collagen, glycosaminoglycans and proteoglycans, which are major components of the ECM [46]. Granulation tissue formation begins to form three-four days after wounding [47]. Collagen I expression is detected in deep dermal fibroblasts in adjacent tissues 16 h after incision of full-thickness wounds [48]. The number of collagen I expressing fibroblast increases until day 6 [48]. Compared to collagen I, activation of collagen III expression activation is slower and is seen in 5-day human wounds [49]. Collagen III expression reaches its maximum in the second-week post-wounding [50]. This corresponds to the remodelling phase of wound healing [51].

Upon injury, fibroblasts start producing and secreting tropoelastin. Tropoelastin is a 60-70kD soluble monomer precursor of elastin [52, 53]. These tropoelastins are secreted into the extracellular space and self assemble through coacervation [54]. These tropoelastins are then crosslinked by lysyl oxidase forming elastin fibres. Although they are deposited during wound healing, these fibres are aberrant and this contributes to reduced elasticity of a scar [55, 56].

It is still unclear which precursor cells myofibroblasts originate from [57]. It is suggested that myofibroblasts may originate from fibroblasts, smooth muscle cells, bone-marrow-derived circulating fibrocytes or mesenchymal stem cells [58, 59]. Fibroblasts transitioning into myofibroblasts begins when they enter the wound environment [60]. They become proto-myofibroblasts and start forming bundles of actin and myosin intracellularly [61, 62]. They then differentiate into myofibroblast, expressing α -smooth muscle actin (α SMA)[63]. Regardless of where myofibroblasts come from, they are responsible for granulation tissue contraction, reducing scar size.

The remodelling phase is a slow process whereby sparse ECM is gradually replaced by denser ECM mainly comprised of collagen [39]. Even 6-weeks post-injury, the newly healed skin, is only ~50% of its original strength [64]. This scar tissue is functionally deficient as it has fewer blood vessels and is devoid of hair follicles and sweat glands [65].

1.4 Pressure ulcers in the world

1.4.1 Pressure ulcer prevalence and incidence

Pressure ulcers (PU), also known as decubitus ulcers, bedsores or pressure sores are the result of pressure maintained over an area for extended periods of time. At its 2007 consensus conference, The National Pressure Ulcer Advisory Panel (NPUAP) defined pressure ulcers as: “localised injury to the skin and/or underlying tissue over a bony prominence, as a result of pressure, or pressure in combination with shear and/or friction” [66]. As such, PU usually develops in areas such as the elbow, heel, hip, shoulder, back and back of the head (Fig. 1.4). This can lead to extended hospitalisation, with a significant financial burden on healthcare services.

People who are susceptible to developing PU include the aged and the immobile. Physical complications resulting from age also increase the risk of developing PU. These include urinary or faecal incontinence, decreased serum albumin levels, decreased diastolic blood pressure, diabetes mellitus, low body weight and decreased dietary intake [67, 68]. People who are prone to PU are mainly residents in nursing homes [69], critical care units [70, 71] and acute care units [72, 73].

Figure 2.4: Figure showing where PUs usually develops. a) Locations where PU develops if the person is lying on their back. b) Locations where PU develops if the person is lying on their side. Adapted from The Straits Times newspaper: Home section, September 8, 2015

A 2008 – 2009 International Pressure Ulcer Prevalence™ survey in the United States (USA), reported overall PU prevalence and facility acquired (FA) ulcer rates were 13.5% and 6% in 2008 (N = 90,398) and 12.3% and 5% in 2009 (N = 92,408)

respectively. More recently, a systematic review of PUs in public hospitals reports a global incidence of 6.3%. In Singapore, PU prevalence was at 18.1% and incidence was 8.1% in 2005 [74]. In a recent publication in 2020, Graves et. al reported that 98.86% of Stage 3 or Stage 4 ulcers originated from the community and only 1.13% was from healthcare institutions, based on data acquired from Singapore General Hospital (N = 1143) [75]. The low occurrence of Stage 3 and Stage 4 ulcers in healthcare settings suggested that Stage 1 and Stage 2 ulcers are better managed in healthcare settings compared to the community setting [75]. Graves et. al also reported declining incidence rates of Stage 3 and Stage 4 PUs from 4.05% to 3.4% per 1000 admissions from 2016 to 2019. The management of PUs requires caregiver time and healthcare services. In the USA alone, it has been reported that each case of a PU can incur a cost of \$500 to \$70,000 USD depending on the severity, amounting to a total of \$11 billion USD annually [76]. Considering that PU can be prevented by patient repositioning, nutritional intervention, special support surfaces, extra skincare and adopting risk assessment tools [77], the incidence and associated costs can be reduced.

There are situations where interventions, like patient repositioning, cannot be carried out. Such as repositioning a critically ill person or patients undergoing prolonged surgical procedures. In these cases, patients are usually positioned in ways that minimise risk. Common surgical positions like supine, prone and kraske/jackknife are adopted with proper support surfaces [78]. In an earlier study in 2002, 44 out of 208 patients who underwent more than four hours of surgery developed 70 PUs (31 Stage 1 PU, 39 Stage 2 or worse PU) in the first 2 days after surgery. Amongst all PUs, 59.2% of them developed on the heels and 15.7% developed in the sacral area [79]. In a more recent study involving patients who had 30 – 280 minutes of surgery, the incidence of

PU was very low (1.3%, n= 1236). However, this low rate may be due to the inclusion of patients undergoing shorter surgeries where pressure-induced ischaemia duration may not be damaging [80].

Pressure ulcers are also associated with increased mortality. Patients with a PU have a 3.6 times higher risk of dying within 21 months compared to those that do not [81]. Another study reported that patients with a pressure ulcer on admission, developing new pressure ulcers during admission or with the pressure ulcer failing to recover, in long-term care had 2-3 times increased risk of dying within six weeks of admission [82]. Despite evidence associating PU with an increased risk of death, it is not classified as a main cause of death. Rather, developing PU is a sign of an underlying disease and this would result in complications and an increase in mortality [81].

1.4.2 Co-morbidities directly associated with pressure ulcers

Pressure ulcers commonly develop in older patients with co-morbidities. Older patients usually experience sarcopenia and frailty characterised by reduced muscle strength, weight loss and immobility [83]. Furthermore, chronic diseases usually plague these older patients [84]. Special attention should be paid to co-morbidities to promote earlier identification of PU. Earlier identification of PU would allow them to be more holistically assessed and better understood.

Cardiovascular disease may result in low cardiac output, hypotension, reduced blood perfusion and peripheral ischaemia contributing to PU progression [85]. In many older patients, atherosclerosis is the primary cause of a compromised vascular system. Well-known risk factors leading to atherosclerosis include smoking, hypertension,

hyperlipidemia, diabetes and a sedentary lifestyle [86]. Atherosclerosis results in decreased blood circulation to essential organs such as the heart, brain, kidneys and skin. This reduced circulation to the skin may contribute to PU development [86]. Diabetes is another common chronic disease as more people age and becomes sedentary [87]. Diabetic patients suffer from vascular complications resulting in local ischaemia caused by glycosylated vessels [88]. Again, local ischaemia in the skin may contribute to PU development.

1.4.3 Current strategies for preventing pressure ulcers

A recent review by Boyko et al. 2018 on the current management of PU, recommended strategies for preventing PU formation and progression by alleviating pressure or placing padding over high-risk areas [31]. Patients are also evaluated for risk of developing a PU with preventive measures put in place. Any high-risk patients would be assigned a PU prevention plan, involving repositioning, to be catered to their needs [31]. However, the frequency of repositioning should also take into consideration the patient's support surface, medical condition, and general fragility of the skin as the process of repositioning may also produce shear forces [31]. When patients are repositioned, it is of importance that there is a constant assessment of the skin as this is key to identifying a Stage 1 PU. This allows immediate intervention such as alleviating pressure from the affected skin to prevent PU progression [31].

Repositioning guidelines suggested patients be repositioned every 2 h. However, there is no evidence to determine if this is the optimal duration to reposition patients [89]. This duration should be determined empirically according to each patient. This repositioning guideline has also been viewed as elderly abuse and is reviewed in a

recent article by Sharp et al., suggesting that instead of repositioning the patient around the clock, all patients should be provided with a pressure changing bed [90]. These pressure changing devices are available to help reduce or relieve pressure without the need for a caregiver. Such devices aim to lower capillary closing pressure of 32 mm Hg and results in lowering PU incidence by 60% when compared to normal mattresses [91].

Other than repositioning the patient, a variety of cushion pads are available to reduce the risk of PU formation [31]. Such cushion pads are designed specifically to cushion over pressure points of high-risk areas on the body such as the heels and sacrum [31]. Proper padding and fitting of prosthetics are also important for patients to cushion tissue prosthetic interfaces and minimise slippage that would create friction [31].

The skin microclimate is also important in the prevention of PU. Sweat, urine, and faecal matter can lead to alterations in skin microclimate and lead to the maceration of the skin [92]. A macerated skin is prone to superficial breakdown and may contribute to the formation and progression of a PU. According to PU prevention guidelines, the skin should be kept dry and cool to reduce the risk of PU formation. Skin dryness is associated with a decreased coefficient of friction and hence reduces damage to the skin in the context of frictional damage [93]. In addition, the drier the skin surface, the less the local skin temperature increases affecting deeper tissues and this may confer protection [94]. Localised skin cooling may confer protection from PU formation [95]. Animal studies show that local skin cooling protects against pressure ulceration [96] and reduces ischaemia compared to non-cooling or heating on affected skin [97, 98]. Cooling of the skin also reduces post-occlusive hyperaemia, which also contributes to

a protective effect [99]. Finally, cooling, compared to heating, also seems to reduce pressure-induced inflammation when compared to heating the skin in rats [100].

A patient's balanced nutrition is claimed to be important in the prevention of PU. Special attention is placed on the patient's protein intake to ensure that there is a positive nitrogen balance. Vitamins and minerals are also recommended in diets [101]. However, a causal relationship between nutrition and the formation of PU has yet to be determined [86, 102, 103]. A Cochrane review established that the relationship between nutrition and PU prevention cannot be proven due to insufficient evidence [103].

To minimise shear forces, the head of the bed should not be elevated to more than 30 degrees and the angle of elevation should be kept to a minimum. This is also true for wheelchairs as 30 degrees of wheelchair tilt is found to be needed to relieve pressure around ischial and sacral areas [104].

1.4.4 Preventing a Stage 1 pressure ulcer from progressing

When the PU progresses into a Stage 2 PU, a partial thickness wound, the epidermal barrier is lost and the patient is at risk of infection. In the hospital, nosocomial infections are a concern especially when resistant bacteria are involved. A study by Allman et al. in 1999 reports that 45.9% (17 out of 37 patients) of patients have PU acquired infections compared to 20.1% of patients without PU (50 out of 249 patients), indicating that patients are at higher risk if they develop at Stage 2 or higher PU [105]. The main interest of this study is to prevent a Stage 1 PU from progressing into a Stage 2 PU. A Stage 1 PU is characterised by its non-blanchable appearance. An intervention could be administered to prevent PU progression from Stage 1 to more severe stages.

1.4.5 Pathophysiology of pressure ulcers

A recent review by Mervis and Phillips (2019), summarises the pathophysiology of PU to three key points [106]. These are sustained pressure over a bony prominence leading to tissue ischaemia and necrosis, a combination of sheer force and friction while lying at an incline contributing to tissue hypoxia, and excess moisture on the skin leading to skin maceration [106].

Tissue ischaemia resulting from shear in combination with friction and prolonged pressure is central to pressure ulcer progression. Damage produced by short periods of ischaemia is reversible if the circulation is restored in time [107]. However, if the duration of ischaemia is too long, tissues sustain irreversible damage and die [107]. Cells undergoing ischaemia will switch their metabolism from aerobic to anaerobic, causing the accumulation of lactic acid which results in lower cellular pH. If this is prolonged, the depletion of glucose and lower pH will lead to cell death [108].

Intracellular ion concentrations are also affected during ischaemia. The lack of ATP causes the inactivation of the Na^+/K^+ ion exchangers and activates the Na^+/H^+ ion exchangers [109]. Due to ischaemia, intracellular H^+ increases and the cells pump H^+ ions out and intracellular Na^+ increases. This leads to the activation of $\text{Na}^+/\text{Ca}^{2+}$ ion exchangers that produces an increase in intracellular Ca^{2+} . Ca^{2+} then accumulates in the cell due to the inactivity of ATP dependant calcium pumps [107]. This calcium accumulation is known to activate cell death pathways such as apoptosis and necroptosis [110, 111]. This increase in calcium is found to be due to reperfusion instead of ischaemia. Smith et al. found that total tissue calcium content was similar in

pre-ischaemia and end ischaemia and only started to increase after reperfusion in the muscle of canine gracilis [112].

1.4.6 Studies using I/R animal models of pressure ulcers

A review in 2007 details the history of animal models of PU from 1961 to 2000 by Houwing et al and Peirce et al [113]. The authors concluded that there has not been a universally accepted animal model and understanding the mechanisms that produce one could remain out of reach [113]. Although there is not a universally accepted PU animal model, the majority of the PU research community accepts that ischaemia reperfusion is the main cause for PU formation.

1.5 Connexin43 in ischaemia reperfusion injury

1.5.1 Brief introduction to connexins

Gap junctions allow the passage of molecules of up to 1 kDa between cells [114]. In vertebrates, connexin proteins are used to form gap junctions. Gap junctions are made up of two connexons (or hemichannels) docking to each other from separate cells (Fig. 1.5). Each connexon is made up of six connexin proteins, which consist of two extracellular loops, an N- and C- terminal, four transmembrane domains (TM1-TM4) and a cytoplasmic loop [115].

Figure 1.5: Figure describing connexins. Images taken from Nielsen et al., 2012. Legend: N-terminal tail (NT), extracellular loop (E1, 2), transmembrane region (TM 1-4), cytoplasmic loop (CL), C-terminal tail (CT).

Connexins are commonly named according to their predicted molecular weight. For example, Cx43 is roughly 43 kDa. Connexins can also be classified into five subgroups (α , β , γ , δ , ϵ) according to their sequence identity and the length of their cytoplasmic loop (CL). Connexins are then abbreviated with GJ, which means gap junction, followed by their subgroup and numerated according to the position of discovery. For example, Cx43 belonging to the α subgroup was discovered first and hence take on the name GJ $\alpha 1$ [114, 116]. There are a total of 21 connexins found in a human and 20 in a mouse, of which 19 of them can be orthologous pairs [114, 117].

1.5.2 Connexin docking

Connexins from different cells dock to form gap junctions by interactions of E1 and E2 with connexins on another cell and were shown first using Cx32 [118]. Blocking either E1 or E2 with peptides recognising amino acids position 39-76 (E1) or amino acids

position 164-189 (E2) was sufficient to prevent Cx32 gap junction formation [118]. Of all the amino acids blocked, the important amino acids are 3 conserved cysteine amino acids in E1 and E2 which form disulfide bonds that are important for functional gap junction channels [118, 119]. The importance of these cysteines in gap junction formation (Cys 53, 60, 64, 168, 173, 179) were shown by the diminishing cell to cell conductances when cysteines were changed to serines [118].

1.5.3 Cytoplasmic tail

The functionality of connexins depends on the interaction of the C-terminal tail and the cytoplasmic loop [120]. For Cx40, 43, 46, the interaction of the C-terminal tail and the cytoplasmic loop acts to close the channel while for Cx26, it is the disruption of the C-terminal tail and cytoplasmic loop interaction that causes it to close [121]. Interestingly, in the case of Cx43 and 46 hemichannels, the interaction of the C-terminal tail and the cytoplasmic loop acts to open the channel instead [120]. This was shown when Cx43 hemichannels lacking their CT tail were not functional [122].

The cytoplasmic tail also interacts with targets such as tubulin [123], CIP85 [124], Drebrin [125], proto-oncogene tyrosine-protein kinase Src (c-Src) [126], phosphatase and tensin homolog (Pten) [127], E3 ubiquitin-protein ligase NEDD4 [128], and Zonula occludens-1 (ZO-1) PDZ domain [129, 130]. The cytoplasmic tail also contains phosphorylation sites for kinases such as Src [131], tyrosine kinase 2 (Tyk2) [132], cyclin-dependent kinase 1 (CDK1) [133], mitogen-activated protein kinase (MAPK) [134], casein kinase 1 (CK1) [135], protein kinase A (PKA) [136], protein kinase C (PKC) [137], protein kinase B (Akt kinases) [129, 138]. Notably, E3 ubiquitin-protein

ligase NEDD4, two MAPK phosphorylation sites Ser279 and Ser282 are involved in the turnover of Cx43 protein levels [128, 139, 140].

1.5.4 Connexins as hemichannels

Connexins may also operate as hemichannels. Although usually closed in normal circumstances, cells undergoing ischaemia-reperfusion or oxidative stress can lead to the opening of hemichannels releasing metabolites and secondary messengers [141-144]. These molecules include adenosine triphosphate (ATP), nicotinamide dinucleotide (NAD), Ca^{2+} , glutamate and inositol 1,4,5-triphosphate (IP3) [141-144]. In the cochlea, IP3 has been reported to be able to travel out of the cell via open hemichannels and activate the release of Ca^{2+} stores in neighbouring cells via activation of IP3 receptors in a paracrine signalling fashion [143]. This can cause the neighbouring cells to die [143]. Calcium signalling can also be activated by the purinergic G protein-coupled P2Y receptor (P2Y) by ATP [145]. Activation of the P2Y receptor by ATP is stopped by depleting ATP, and apoptosis is reduced, indicating that ATP can also induce neighbouring cells to die. ATP can also activate ATP-gated P2X (P2X) receptors, which also results in apoptosis. However, inhibition of P2X receptors only partially prevents apoptosis caused by ischaemia-hypoxia in cardiomyocytes [146].

1.5.5 Skin connexins

In the skin, gap junctions are expressed abundantly. Notably, different types of connexins are expressed at different levels in the different layers of the epidermis and the dermis. Connexins found in the skin of humans and mice include Cx26, Cx30, Cx30.3, Cx31, Cx31.1, Cx37 and Cx43 [147, 148]. During wounding and skin repair,

connexin expression changes at the wound edge, indicating that connexins play roles in wound healing [149]. In healthy unwounded skin, certain connexins such as Cx26, 30 and 43 are expressed in the epidermis of mice [147]. Connexin43 is expressed in the stratum spinosum and stratum basale while Cx26 and Cx30 are expressed mainly in the stratum granulosum [147]. In the human epidermis, Cx43 is mainly expressed in the suprabasal layers where the highest expression can be found in the granular layer [147]. Connexin43 is also expressed in fibroblasts [149, 150]. In an open wound, connexin levels change in different stages of wound healing. In rodents, one day after wounding, Cx26 and Cx30 expression are turned on in all layers of the epidermis at the wound edge and turned off when the wound re-epithelializes [149]. Connexin43 levels are greatly reduced in the epidermis at the wound edge but up-regulated in the proliferating cells behind the leading edge [149]. In diabetic rats, epidermal Cx43 was reduced compared to the skin of non-diabetic rats [151]. However, epidermal Cx43 was significantly increased in diabetic rat wound edges when compared to normal rat wounds, where epidermal Cx43 was reduced [151]. The rate of re-epithelialization was also significantly reduced [151]. This was further supported when the diabetic rat wounds healed more quickly when treated with Cx43 antisense oligonucleotides (Cx43asODN), showing that decreased Cx43 expression improves diabetic wound healing [151].

1.5.6 Connexin43 and genetic mutations

Connexins are important for the normal functions of many organs and mutations in the connexins can cause diseases. More than 70 different missense mutations in a single copy of GJ $\alpha 1$ or Cx43 are associated with Oculodentodigital dysplasia (ODDD) [152, 153]. Among the 70 mutations, Cx43D3N and V216L fibroblasts have reduced gap

junction communication, proliferation and migration [154]. Mutations Cx43L7V, G138R, G143S showed slower proliferation and migration as well as abnormal gap junction plaque formation, phosphorylation and hemichannel activity [155]. Mutations can also affect other connexins in a dominant-negative fashion. In one example, Cx31G45E mutation stopped Cx43 from reaching the plasma membrane in HaCaTs and HeLa cells and resulted in necrotic cell death [156]. The above examples are not exhaustive and there are many other examples of connexin mutations being linked to disease [157].

1.5.7 Connexin43 in chronic wounds

In human chronic wounds, diabetic ulcers, venous leg ulcers and pressure ulcers, all have Cx43 up-regulated around the wound edge [158]. For venous leg ulcers, epidermal Cx43 was eightfold higher 1 mm from the wound edge than in patients intact control skin biopsies [158]. Epidermal Cx43 expression upregulated 14-fold as we move further away from the wound edge [158]. An accompanying observation was that the epidermis became hyperthickened with increasing epidermal Cx43 expression [158]. Dermal Cx43 also increased 20-fold at the wound edge and 32-fold at the far edge when compared to matched unwounded tissues [158]. In diabetic ulcers, epidermal Cx43 was elevated ninefold at the wound edge and sevenfold in the epidermis further away [158]. Similar to venous ulcers, dermal Cx43 was increased 20-fold at the wound edge and 18-fold away from the wound edge [158]. Finally, for pressure ulcers, epidermal Cx43 increased 10-fold 1mm from the wound edge [158]. Dermal Cx43 was increased 57-fold at the wound edge and 37-fold away from the wound edge [158]. Mendoza et al. observed increases in dermal N-cadherin, Zonula Occludens-1 (ZO-1) and Cx43 in human venous leg ulcers [159]. They also reported that when treated with Cx43asODN,

lower Cx43 expression also resulted in lower expression levels of N-cadherin and ZO-1 in fibroblasts [159]. Down-regulation of Cx43 and N-cadherin but not ZO-1 expression in-vitro resulted in faster cell migration with longer lamellipodial protrusions at the leading edge [159]. Mori et al. report that when Cx43 is down-regulated with antisense in acute murine wounds there is reduced inflammation, reduced Ccl2 and TNF α [160]. Active Cx43 down-regulation resulted in increased keratinocyte proliferation at the wound edge. Enhanced fibroblast migration was also observed during wound healing [160]. Further evidence was also provided by Xu et al. when *S. aureus* exudate was used on HaCat cells to understand what pathways were involved [161]. Cx43 was found to be upregulated in migrating HaCat cells exposed to *S. aureus* exudate and showed slower migration [161]. Furthermore, Akt and ERK1/2 showed upregulated phosphorylation levels during HaCat cell migration and when Akt phosphorylation was inhibited, wound healing was accelerated [161]. In addition, Cx43 upregulation was also inhibited when an Akt inhibitor was used [161].

1.5.8 Cx43 and ischaemia reperfusion injury

Chronic wounds are mainly categorised into diabetic foot ulcers, venous leg ulcers and pressure ulcers [17]. In all three, there are repetitive ischaemia reperfusion events [16]. As discussed in section 1.5.1, ischaemia reperfusion can lead to the formation of a PU.

Following a stroke, Cx43 is up-regulated in the astrocytes in the brain [162-164]. Furthermore, gap junctions remain open during ischaemia and continue to remain coupled during the process of cell death in astrocytes [165]. It has been reported that inhibiting gap junction communication can reduce neuronal death caused by a stroke

[164, 166]. Gap junctions have roles in the spread of death signals in what has been termed the “bystander” effect [167]. With the inhibition of Cx43, the spread of such death signals from initial lesions are also inhibited, reducing neuronal death.

In the heart, Cx43 is involved in the electrical coupling of cardiomyocytes [168-171]. Connexin43 is also implicated in reperfusion injury in myocardial cells and the injury is reduced by selectively blocking Cx43 hemichannels [172, 173]. Connexin43 also plays important roles in retinal ischaemia. In a rat retina ischaemia-reperfusion model, Cx43 is increased in the ganglion and nerve fibre layer, likely to belong to retinal astrocytes and Muller cells [174]. Cx43 is also increased in retinal vascular endothelium after elevating intraocular pressure for 60 min in Wistar rats [174]. Retinal I/R injury was reduced by injecting Cx43 mimetic peptides intravitreally [175, 176], and by reducing vascular hyper-permeability [176]. Down-regulation of Cx43 by Cx43 antisense oligodeoxynucleotide (Cx43asODN) was also able to reduce inflammatory responses in organotypic cultures of optic nerves which underwent 2 h of ischaemia and 2 h to 6 d of reperfusion [177].

Cx43 is upregulated, after spinal cord injury (laminectomy of the eighth thoracic vertebra) reaching three times higher than normal levels at 4 weeks [178]. The upregulation of Cx43 in astrocytes is also confirmed in another model of chronic constriction injury (CCI) of the sciatic nerve citing an increase of Cx43 for more than three weeks [179]. Administration of Cx43 mimetic peptide was able to block Cx43 communication and to elicit the recovery of the spinal cord in rats [180]. In another spinal cord injury model (laminectomy on the C5 vertebra), blocking Cx43 expression with Cx43asODN was found to be able to reduce glial cell activation and neutrophil

recruitment into the injury site. Treatment also resulted in reduced vessel hyper-permeability. Spinal tissues showed less tissue swelling, distortion and disruption [181].

By looking into ischaemia reperfusion studies in many tissues, evidence shows that Cx43 plays an important role in the “bystander” effect. Cx43 is important in skin development and homeostasis and it is very likely to also play a role in the development of PUs [149, 158].

1.5.9 Connexin bystander effect

As previously mentioned, gap junctions allow small molecules of up to 1 kDa of molecular mass such as secondary messengers and metabolites to pass through them. These include ATP, ADP, Ca^{2+} , glutathione and many others [182, 183]. The bystander effect was reported in 1998 by Lin et al. by using cells that overexpressed anti-apoptotic bcl2 protein and Cx43 ($\text{bcl}^+\text{Cx43}^+$), making the cells resistant to apoptosis and have the ability to couple with other cells via Cx43 gap junctions [184]. When these cells were challenged with calcium ionophore, less than 5% of $\text{bcl}^+\text{Cx43}^+$ cells grown in homogenous cultures died [184]. In contrast, bystander killing was shown when the apoptosis-resistant $\text{bcl}^+\text{Cx43}^+$ cells died when coupled to non-bcl2 expressing Cx43^+ cells when challenged with calcium ionophore [184]. In another publication by Sanson et al., Cx43-mediated bystander killing was demonstrated by showing that treating a Cx43 transfected co-culture of 30% thymidine kinase positive cells (TK^+) and 70% TK^- cells with ganciclovir resulted in 20-30% cell mortality when a gap junction blocker,

18- α -glycyrrhetinic acid, was used [185]. In contrast, 100% mortality was observed when no 18- α -glycyrrhetinic acid was added [185].

However, different connexins have certain preferences and selectivity to molecules [114]. For example, Cx43 channels have a high cation preference as compared to Cx32, which has a slight anion preference [186-188]. The high cation preference of Cx43 leads to the suspicion that Ca^{2+} plays a major role in gap junction related functions such as the bystander effect [184, 189]. Abnormal levels of intracellular Ca^{2+} can lead to cell death by apoptosis, autophagy, anoikis and necrosis [190, 191]. In apoptosis, Ca^{2+} ions mainly play roles in the intrinsic pathway involving the transient release of Ca^{2+} from the endoplasmic reticulum and subsequent transmission to mitochondria. This accumulation of Ca^{2+} in the mitochondria leads to destabilisation and permeabilisation of the mitochondria membrane, thus releasing pro-apoptotic factors such as cytochrome C [192]. In necrosis reported in pancreatic cells, sustained increased Ca^{2+} levels can inhibit mitochondria function, which leads to ATP depletion. This causes the activation of pancreatic digestive enzymes that result in necrosis of the pancreatic acinar cells [192, 193]. Recently, another cell death pathway, necroptosis or regulated necrosis has been recognized [192, 194]. Not surprisingly, Ca^{2+} influx from the external environment after mixed lineage kinase domain-like protein (MLKL) membrane localisation and trimerisation are important for TNF-induced necroptosis [195]. In another publication, cytosolic accumulation of Ca^{2+} and continued c-Jun N-terminal kinase (JNK) activity triggered necroptosis [110].

1.6 Research gaps

Stage 1 pressure ulcers are a potential target for treatments that could improve their healing and stop them from progressing to a Stage 2 ulcer and beyond. Currently, there are preventive interventions like repositioning, introducing specialized support surfaces, nutrition and applying dressings [196, 197]. Despite established preventive strategies, PUs remain a common problem [198]. A literature review of Stage 1 PU revealed that there is a general lack of information regarding early processes leading to the progression to a Stage 2 PU. This is further supported by the lack of therapeutic interventions specifically aimed at preventing PU progression of Stage 1 PU [196, 197]. There is a need to identify and understand what happens in an early PU and this can be done by optimising and validating an animal model to study what happens to a developing PU and aim to intervene during the early PU development to attempt to prevent ulcer progression.

1.7 Hypothesis

My hypothesis is that the initial cause of PU formation starts with ischaemia reperfusion injury with Cx43 expression contributing to cell death and inflammation.

1.8 Aims

To test my hypothesis, a mouse model has to be designed to recapitulate a Stage 1 PU. This would allow the study of the local processes happening in the skin. An extension to this model involving an additional I/R cycle will be used to generate a Stage 2 PU. We will then test if downregulating Cx43 in a Stage 1 PU with an additional I/R cycle will prevent progression to a Stage 2 PU.

Aims:

- Establishing a mouse model where
 - There is an I/R injury that is non-blanchable where the epidermis is still intact
 - Parameters should include the highest possible ischaemia duration that does not result in an open wound.
- After establishing a model in aim 1, aim 2 will include characterising the histological, Cx43 and inflammatory changes.
 - Qualitative observations from histology
 - Quantifying epidermal Cx43
 - Qualitative observations of vessel hyperpermeability

Chapter 2. Methods and Materials

2.1 Mice

ICR-1 (IcrTac: ICR) and C57/B6 mice (Invivos, SG) were used in this study with approval from the Institutional Animal Care and Use Committee (IACUC) under the IACUC number 140928 and A0367. Only male mice were used in experiments. Procedures were performed when they were 7-12 weeks old. Mice were randomly sorted into groups if needed (e.g., Vehicle controls vs. Treated).

Anaesthetic and Analgesia: Before any procedures, subcutaneous buprenorphine (2 mg/kg) was administered. This provides pain relief for 12 h. Further doses were administered if any ongoing signs of discomfort were detected. Anaesthesia induction was achieved by 4% isoflurane and 2 L/min oxygen in a chamber. Anaesthesia was maintained by transferring mice onto a nose cone with the same concentration.

2.2 Transgenic mice

All transgenic mice purchased and bred for this study is with the approval from the Institutional Animal Care and Use Committee (IACUC) under the IACUC numbers E0029 and A18045. Experiments performed on these transgenic mice were also approved under IACUC no. 140928 and A0367. Transgenic mice to knock out Cx43 in endothelial cells or neutrophils. The transgenic mice, B6.129S7-Gja1tm1Dlg/J (Cx43^{flx/flx}), with flanking loxP sequences around Cx43 were obtained from The Jackson Laboratory, USA [199, 200].

Neutrophil Knockout mice: To achieve cell-specific knockout in neutrophils, B6.129P2-Lyz2tm1 (Cre)Ifo/J (LysMCre) mice where expression of Cre recombinase is driven by the lysM promoter and will only be expressed in cells expressing lysozyme [201, 202] (Fig 2.1a).

Endothelial Knockout mice: To achieve conditional and inducible knockout in endothelial cells, Tie2ERT2Cre mice (Tie2Cre) (Tg (Tek-Cre/ERT2) 1Arnd, EM:00715) were purchased from The European Mouse Mutant Archive (EMMA). In these mice, Cre recombinase will only be expressed in endothelial cells, as the Tie2 promoter driving the TEK gene is exclusively active in endothelial cells. Cre recombinase will only be exported into the nucleus to perform recombination when tamoxifen is administered in these mice [203] (Fig. 2.1b).

Figure 2.1: Differences in between Tie2CreER and LysMCre activations. a) Figure showing an example for a cell-specific Cre expression in a neutrophil expressing (1) Cre recombinase and (2) translation into the cytoplasm. Cre recombinase enters the nucleus, recognizes the LoxP sites and cleaves the gene leaving one LoxP site. b) Figure showing an example of an inducible cell-specific Cre expression in an endothelial cell expressing (1) Cre recombinase-oestrogen receptor (CreER) with a mutated ligand-binding domain and (2) translation into the cytoplasm. (3) Upon tamoxifen administration, tamoxifen enters the cell and binds to CreER, which allows the complex to (4) enter the nucleus where recombination occurs.

2.3 Transgenic mice breeding and mating.

All breeding of animals was approved by the Institutional Animal Care and Use Committee (IACUC). Briefly, Cx43^{flx/flx} mice are mated with LysMCre mice and Tie2Cre mice to generate F1 generation consisting of double heterozygous for both genes. These F1 offspring were then mated back to Cx43^{flx/flx} mice to generate mice homozygous for Cx43^{flx/flx} and heterozygous for the Cre gene. These mice were then mated with Cx43^{flx/flx} to generate a mixture of experimental and control animals (Fig. 2.2).

Figure 2.2: The breeding strategy of transgenic mice lines. a) Cx43^{flx/flx} mice were mated with Tie2Cre mice to produce mice heterozygous for both genes with 100% probability. b) Mice heterozygous for both genes were then mated to Cx43^{flx/flx} mice to generate mice homozygous for Cx43^{flx} with a signal copy of Tie2Cre with a probability of 25%. c) The mice were mated again with Cx43^{flx} mice to generate d) experimental animals with their controls. The same strategy is used to generate Cx43^{flx/flx} LysMCre mice.

2.4 Tamoxifen regime

Tamoxifen (T5648, Sigma) was prepared by dissolving in 100% ethanol at 55°C. Corn oil (C8267, Sigma) was later added when tamoxifen was fully dissolved to give a final concentration of 20 mg/ mL. To induce Cre recombination in Cx43^{flx/flx}Tie2Cre mice, 100 μ L of tamoxifen in corn oil was delivered by intraperitoneal injection daily for 5 days. Animals were weighed daily, and animals were allowed to recover for at least a week before any experiments were performed.

2.5 Genotyping

To determine the genotype of the transgenic mice, crude DNA extraction followed by PCR was performed. Approximately 5 mm of tissue from the tip of the tail was collected and lysed overnight in 300 μ L tail lysis buffer (100 mM Tris-HCl, pH 8.8, 5 mM EDTA, 0.2% SDS, 200 mM NaCl, 2 μ g/ mL proteinase K) at 55°C. Proteinase K was then inactivated by incubating tubes at 95°C for 10 min. Tail lysates were then centrifuged at 15000 rpm (Eppendorf centrifuge 5424R) to spin down any remaining debris. 1 μ L of supernatant was then diluted 30x to make a DNA stock. Next, the master mix was prepared as described below (Table 2.1). Samples were then added into the thermal cycler for PCR following denaturing at 90°C, annealing at 65°C for Cx43^{flx}, 65°C for Tie2Cre and extension at 72°C for 30 cycles. After PCR was done, samples were loaded on a 1.5% agarose gel with Sybr safe DNA stain to check for DNA transcripts.

Table 2.1: Constituents of PCR reaction mix with primer sequences.

Reagents (Invitrogen)	Tie2Cre	Cx43 ^{flx/flx}
10x PCR reaction buffer	2 µL	2 µL
MgCl ₂ (50mM)	0.8 µL	0.8 µL
Forward primer (IDT)	0.2, 0.2 (100 µM) Transgene: 5' GCG GTC TGG CAG TAA AAA CTA TC 3' Internal control: 5' CTA GGC CAC AGA ATT GAA AGA TCT 3'	0.1 (200 µM) 5' CTT TGA CTC TGA TTA CAG AGC TTA A 3'
Reverse primer (IDT)	0.2, 0.2 (100 µM) Transgene: 5' GTG AAA CAG CAT TGC TGT CAC TT 3' Internal control: 5' GTA GGT GGA AAT TCT AGC ATC C 3'	0.1 (200 µM) 5' GTC TCA CTG TTA CTT AAC AGC TTG A 3'
DNTP (10mM)	0.4 µL	0.4 µL
Taq polymerase	0.1 µL	0.1 µL
DNA	1 µL	1 µL
Water	14.9 µL	15.5 µL
Total	20 µL	20 µL

2.6 Pressure ulcer mouse models

2.6.1 Standardisation of terms used throughout the thesis

Magnet pinch model – Applying magnets to create ischaemia reperfusion through pinching the skin.

Magnet implantation model – Implanting a magnet or magnetic material under the skin and applying a magnet to the external side of the skin.

Terms used for ischaemia reperfusion durations:

1.5 h(I) – 1.5 h of ischaemia

24 h(R) – 24 h of reperfusion

1.5 h(I) / 24 h(R) – 1.5 h of ischaemia followed by 24 h of reperfusion (single pinch).

1.5 h(I) / 24 h(R) / 1.5 h(I) / 3 d(R) – 1.5 h of ischaemia, 24 h of reperfusion, 1.5 h ischaemia and 3 days of reperfusion

2.6.2 Use of ICR-1 mice for definitive studies and C57/black6 Cx43^{flx/flx} mice for pilot studies

ICR-1 mice were used in both pilot studies for setting up single pinch models and subsequent experiments in Chapter 4 while C57/black6 Cx43^{flx/flx} mice were used in the setting up of double pinch models, studying subcutaneous administration of 30% pluronic gel, Cx43asODN diffusion studies into the skin and used in subsequent experiments in Chapter 5.

2.6.3 Mouse magnet single pinch ischaemia reperfusion model

This model used a pair of magnets to pinch the skin on the back of the mice, leaving two injury areas in the skin (Fig. 2.3) [204]. While the mice were under anaesthesia, the back of the mice was shaved, and depilatory cream applied. The back was wiped clean with warm water. Next, the skin was gently tented, forming a fold and the magnets (Gold plated neodymium magnets; F656S, 12 mm diameter x 2 mm, 2000 Gauss, N42; Magnet Expert Therapy Magnets, UK) were applied, leaving a 1 cm skin bridge. Skin, including epidermis, dermis, subcutaneous fat layer, panniculus carnosus and subcutaneous loose connective tissue layer, were pinched. Mice were returned to non-magnetic cages. After the ischaemic process, the magnets were removed to allow blood to reperfuse causing reperfusion injury. All ischaemia and reperfusion timings are recorded in Table 2.2.

Figure 2.3: Pictures of magnet single pinch model. a) Dorsal skin of mice shaven with the hair removed. The skin was tented, and magnets were applied on both sides. b) Mice were allowed to return to their cages for a period of ischaemia. c) Close up image showing blood vessels are visible in surrounding skin. d) Immediately after magnets are removed, skin shows discoloured phenotype. e) A few minutes later, blood was seen to return in the major vessels.

Table 2.2: Ischaemia and reperfusion durations for single pinch experiments

Description of experiment	Ischaemia timing	Reperfusion timing
Comparing magnet models	1.5 h	24 h
Finding suitable ischaemia timing	1.5 h, 2 h, 2.5 h, 3 h	24 h
Checking for wound appearance using 1.5 h of ischaemia	1.5 h	8 days
Observing histology events in 1.5 h of ischaemia	1.5 h	4 h, 24 h, 4 days and 8 days
Observing macroscopic appearance of drug administration	1.5 h	4 h and 24 h
Studying Cx43asODN administration	1.5 h	15 min and 8 h

2.6.4 Mouse magnet double pinch ischaemia reperfusion model

Following the single pinch of 1.5 h of ischaemia followed by a period of reperfusion, magnets were reapplied to the previously pinched area for another 1.5 h of ischaemia and then removed for the last period of reperfusion (Fig. 2.4) [204]. Various reperfusion timings for different experiments are recorded in Table 2.3.

Figure 2.4 Pictures of mice magnet double pinch model. a) Dorsal skin of mice with the hair removed. The skin was tented while waiting for magnet application. b) Magnets were applied, and mice were allowed to return to their cages for a period of ischaemia. c) Immediately after magnets are removed, skin shows discoloured phenotype. d) Magnets were reapplied to the same area for the 2nd cycle of ischaemia. e) 2nd removal of magnets.

Table 2.3: Ischaemia and reperfusion durations for double pinch experiments

Description of experiment	1 st Ischaemia	1 st reperfusion	2 nd ischaemia	Final reperfusion
Comparing different reperfusion timings	1.5 h	1.5 h or 24 h	1.5 h	14 days
Observing histology events after double pinch	1.5 h	4 h	1.5 h	24 h
Observing macroscopic appearance of drug administration	1.5 h	4 h	1.5 h	14 days

2.6.5 Magnet implantation model

This model involved the surgical implantation of one magnet under the skin and the application of another magnet on the surface of the skin (Fig. 2.5). This introduced pressure on the dorsal skin on the back of the mice, leaving one insulted area on the back of the mice [205].

While the mice were under anaesthesia, the backs of the mice were shaved, and depilatory cream was applied. The back was then wiped clean of all cream with warm water. An incision was made on the back skin for the magnet (same type as in section

2.6.1) to be inserted to the side of the mouse. The incision was sutured using 4/0 non-absorbable silk sutures (C0762083, Aesculap). The other magnet was then applied to introduce the ischemic insult and to hold the magnet in place. The same skin layers were compressed as described in section 2.6.1. Mice were housed in non-magnetic cages. After 1.5 h of ischaemia, the outer magnet was removed to allow blood to return for 24 h, thus causing reperfusion injury.

Figure 2.5: Magnet implantation model. a) An incision on the dorsal skin was made. b) Blunt tip scissors were inserted to gently create a pocket by shifting the fascia away. c) Magnet was pushed into the incision and d) positioned into the pocket. e) An external magnet was applied to create the pinch for 1.5h. f) The incision was sutured up. g) External magnet was removed. The internal magnet was left in the animal until the time of harvest.

2.7 Cx43asODN in 30% w/v pluronic administration

First, to prepare 30% w/v pluronic gel, 30 g/100 mL of Pluronic® F-127 (Sigma) was weighed out and dissolved in sterile Milli-Q® water overnight at 4°C. Cx43asODN of sequence 5'-GTAATTGCGGCAGGAGGAATTGTTTCTGTC-3' was used (Sigma). The Cx43asODN was first reconstituted in sterile Milli-Q® water to a stock concentration of 3 mM. This is followed by a 10x dilution in 30% w/v pluronic gel to

achieve a final working concentration of 300 μM . To ensure proper coverage of the injury, 100 μL of the Cx43asODN preparation was injected subcutaneously. Care was taken during administration to prevent any additional injury. A 27-gauge needle was inserted close, but not through, the injury site with the bevel facing downwards and adjusted under the skin to deposit the treatment as close as possible to the centre of the injury (Fig. 2.6). The pluronic gel is liquid at 0°C - 4°C , it increases in viscosity as it comes to room temperature so all pluronic and instruments are kept on the ice up till the period of administration.

Figure 2.6: Strategy for drug administration. The needle was inserted away from the injury site (initial site of entry) and moved towards the injury site (site of administration). The drug was then administered in the target area.

2.8 Daily monitoring

Photographs of mice were taken daily for documentation, scoring and analysis. Pictures include the field of view of the overall skin, whole mouse including tail marking and insulted skin.

2.9 Scoring

Pictures were taken every day and adjusted for size before comparison. The scoring method was chosen because there are multiple parameters (size, depth and redness) that were taken into consideration. Pictures are first assessed using size and depth with larger size and depth taking a higher severity score. If two wounds are of similar size and depth, a deeper red would take the higher severity score.

A severity score was constructed to incorporate both sizes and wound redness. *Severity Scoring Calibration Scale*: A severity score from 1 to 7, (with 7 being the most severe), was devised to help assessors determine the severity of the injury (Fig. 2.7).

Healing Calibration Guide: As these injuries eventually healed up, I also provided a healing guide to calibrate scorers' expectations of what the range of healing looked like in the mice. This was needed because initial scoring showed exaggerated scores caused by misidentification of scars as wounds (Fig. 2.7).

Assessment process: Picture sets were coded to blind the scorer to the treatment group. Three independent scorers then ranked the pictures in order of severity. Data were then plotted for a two-way ANOVA analysis. Data were then transformed to be compatible for Kaplan Meier analysis by setting a certain severity score based on their outward appearances to recognize events such as wound opening (severity score 3) and wounds being completely healed (severity score 1).

Figure 2.7: Score system based on macroscopic appearance. The scoring system includes a gradient from 1 to 7 with 7 being the most severe. A healing guide is included to help scorers to differentiate a progressing wound from a healing wound. The macroscopic guide includes both size and “redness” to decide severity while the healing guide informs the scorer to differentiate a scar from a less severe macroscopic appearance.

2.10 Injection of FITC-BSA

2.10.1 Tail vein injection of FITC-BSA

Fluorescein isothiocyanate conjugated with bovine serine albumin (FITC-BSA, Sigma, USA) was used to study vascular leakage. The FITC-BSA was dissolved in 1x PBS to a final concentration of 1.5% w/v. Thirty minutes before euthanasia, the mice were put into a restrainer with their tails exposed. Heat lamps were used to mildly heat the tail to help dilate the veins for easier identification and administration of 200 μ L of FITC-BSA.

2.10.2 Retro-orbital (RO) injection of FITC-BSA

As there is a high fail rate of FITC-BSA injection via the tail vein, RO injection of FITC-BSA was used for later experiments. Retro-orbital injections of 200 μ L of 1.5% w/v of FITC-BSA in 1x PBS were performed 1 h before euthanasia for proper

perfusion of the microvasculature [206]. Mice were anaesthetized using 4% isofluorane and 2 L/min oxygen in a chamber. They were then transferred to a nose cone with their eyes fully exposed for RO injection. Eyelids were pulled back and a 30-gauge needle was inserted into the retro-bulbar space with the bevel facing away from the eye. Mice were returned to their cages for an hour before euthanasia.

2.11 Tissue harvesting and processing.

Immediately after euthanasia by CO₂, insulted skin and a piece of normal skin were harvested and placed on wax cardboard to keep the skin flat. The skin was bisected, with one half stored in 4% paraformaldehyde (PFA) for immuno- and histological staining and another half stored in RNALATER™ for any mRNA analysis. Tissues in RNALATER™ were stored in -80°C. Skin tissues planned for cryo-sectioning were fixed in 4% PFA for 24 hours and transferred into 20% sucrose in 1x PBS and stored at 4°C. Skin tissues planned for paraffin sectioning were fixed in 4% PFA for 24 h and transferred into 70% ethanol and stored at 4°C.

2.12 Tissue embedding and sectioning.

2.12.1 Cryosection

Tissues were embedded in optimal cutting temperature (O.C.T) compound (VWR international, USA) and subsequently frozen using liquid nitrogen. Embedded tissues were then stored at -20°C before cryosectioning using a Leica CM 1900 UV cryostat (Leica, Germany) at 10 µm thickness. Tissue sections were picked up onto polylysine coated slides and stored at -20°C.

2.12.2 Paraffin sections

Tissues were processed using a HistoCore Pearl tissue-processing machine. Tissues were placed into cassettes to be processed and loaded into the processing machine. Tissues were then put through an ethanol concentration gradient (70%, 80%, 95%, 100% x 3, 45 min, 45°C) followed by xylene (3 x 45 min, 45°C) and paraffin (3 x 45 min, 62°C). Tissues in cassettes were then immediately transferred to a tissue embedder (HistoCore Arcadia C and H, Leica, Germany) to be paraffin embedded. Paraffin blocks were then sectioned at 5 µm thickness using a microtome (Leica RM2245, Germany).

2.13 Haematoxylin and Eosin staining

To study the histology of injuries, paraffin sections of 5 µm thickness were stained with haematoxylin (Sigma, USA) and eosin (Sigma, USA) with a Leica Autostainer XL (Leica, Germany). Slides were immersed twice in Clearene™ (Leica Biosystems, Germany) for 5 min then transferred through a gradient of ethanol (100%, 2 min x 2, 95%, 2 min x 2, 70%, 2 min x 2) followed by 2 min in water. Slides were stained with haematoxylin for 1 min and washed in water for 3 min, followed by 0.3% acid alcohol (70% ethanol, 0.3% Concentrated HCl). Slides were washed again with water for 2 min and transferred into Scott's tap water (0.2 mM NaHCO₃, 0.16 mM MgSO₄ in water) for another minute and back into the water for another minute. They were then transferred into 70% ethanol for 2 min and stained with Eosin for 30 s and transferred into 70% ethanol to wash off excess eosin for 2 min followed by 95% ethanol (2 min x 2) and then 100% ethanol (2 min x 2) before transferring into Clearene™ (Leica Biosystems, Germany) for 5 min to dehydrate the tissue. Excess Clearene™ was allowed to dry

before mounting the slides with organo limonene mounting media (Sigma, USA) and coverslipped.

2.14 Dewaxing, antigen retrieval and immuno-staining

2.14.1 Paraffin sections prior to immuno-staining

2.14.1.1 De-waxing

Slides were immersed twice in Clearene™ (Leica Biosystems, Germany) for 5 min then transferred through a gradient of ethanol (100%, 5 min x 1, 95%, 5 min x 1, 70%, 5 min x 1) followed by 10 min in water.

2.14.1.2 Sodium citrate antigen retrieval

After dewaxing, slides were submerged into a slide container filled with 0.01 M citrate buffer (10 mM Sodium Citrate, 0.05% Tween20, pH 6) at 60°C overnight in a water bath. The slide container was then removed and allowed to cool for 10 min at room temperature. Slides were then washed with 1 x PBS for 5 min.

2.14.1.3 ProteinaseK antigen retrieval

To prepare the ProteinaseK working solution, TE buffer (50 mM Tris Base, 1 mM EDTA, 0.5% Triton X-100, pH 8.0) is used to dilute a 20x proteinase K stock solution (400 µg/ ml in 50% glycerol in TE buffer) to 1x. After dewaxing, slides were submerged into a slide container containing ProteinaseK working solution at 37°C for 5 min in a water bath. The slide container was then removed from the water bath and allowed to cool for 10 min at room temperature. Slides were then washed with 1x PBS for 5 min.

2.14.2 Immunostaining

After slides were de-waxed, antigen retrieved, they were allowed to equilibrate to room temperature for 10 min. Tissue sections were then rehydrated by placing them in a small slide holder containing 1x PBS for 10 min, permeabilised using 0.2% Triton X-100 in 1x PBS for 15 min at room temperature and blocked for 1 h in 1% BSA in PBS. Next, they were stained with the respective primary antibodies (Table 2.2) overnight at 4°C and washed with 1x PBS 3 times. They were incubated for an hour with the respective secondary antibodies (Table 2.2) at room temperature and washed for 5 min in 1x PBS. DAPI stain was then applied at 1:10000 dilution for 10 min and washed 2 times with 1x PBST and 2 times with 1x PBS. Finally, coverslips were mounted using Citifluor™ (Citifluor Ltd, UK) and sealed with nail varnish.

Table 2.4: Table of information of primary and secondary antibody used in immunohistochemical stains.

No.	Primary Antibody name	Cat no. Company	Cryosections /Paraffin sections	Antigen Retrieval	Dilution
1	Rabbit anti-Connexin43	C6219, Sigma	Cryosections/Paraffin	N/A	1:1000
2	Rat anti-mouse Ly-6G	127602, Biolegend	Paraffin sections	Proteinase K	1:500
3	Rabbit anti-HMGB1	Ab79823, Abcam	Paraffin sections	Sodium Citrate	1:500
4	Rabbit anti-RIP3	Ab56164, Abcam	Paraffin sections	Sodium Citrate	1:300
5	Rabbit anti-cleaved caspase3	9661L, Cell signalling	Paraffin sections	No Antigen retrieval	1:1000

No.	Secondary Antibody name	Cat no. Company	Dilution
1	Goat anti-rabbit AF488	A11008, Thermo	1:500
2	Goat anti-rabbit AF555	A21428, Thermo	1:500
3	Goat anti-rat AF568	A11077, Thermo	1:500

2.15 Whole mount staining of blood vessel

After harvesting major blood vessels around the heart, vessels were immersed in 4% PFA for overnight fixation. Fat and other tissues surrounding the blood vessels were then carefully removed under a dissection microscope. Vessels were then cut open and pinned on a Sylgard gel in a petri dish using metal pins. The whole process was performed with the vessels immersed in 1x PBS. After the blood vessels were prepared, they were blocked for 2 h in 0.5% Triton X-100 and 1% BSA in 1x PBS. Next, the vessels were stained with 1:1000 Cx43 primary antibody overnight at 4°C. Three washes were performed using 0.5% Triton X-100 and 1% BSA in 1x PBS and incubated with goat anti-rabbit AF488 for 2 h. This was followed by two washes of 0.5% Triton X-100 and 1% BSA in 1x PBS. The tissues were counterstained using 1:10000 DAPI diluted in 1x PBS for 15 min and washed two times using 1x PBS. Tissues were immersed in 1x PBS and stored at 4°C until imaging.

2.16 Imaging

2.16.1 Tissue slides

All confocal images were taken with a Leica SP8 upright confocal microscope (Leica, Germany) using a 40x objective (506358, 1.3NA, oil, PL, APO, Leica). Confocal

images were taken in 1024 by 1024 format with each pixel having 8-bit depth. Whenever possible, three fields of view of 3 μm stacks were taken for image analysis. These 3 μm -stacked images are made up of 5 single planes of depth 0.6 μm and processed using max projection on the LasX software (Leica). To determine where the halfway point is in the tissue, the top and bottom limit of the tissue is identified on the z-axis. After the top and bottom limit is known, the middle can be identified. The first field of view will be taken at the centre of injury. Subsequent fields of view were taken one field of view away from the centre to eliminate bias. In the event where the epidermis is severely damaged or close to necroinflammatory debris (NID), the field of view was adjusted away from the NID but kept within the central region. All positions are first selected using the DAPI signal to prevent any bias.

Histological images and montages were taken using an Axioscan.Z1 slide scanner (Zeiss) using a 20x objective. Individual images showing key observations were then extracted from the montage using the Zen software (Zeiss).

2.16.2 Whole mount

Confocal images of whole-mount vessels were taken with an HCX APO 40X long working distance dipping objective (506155, 0.8NA, water, APO, Leica). Three fields of view per sample were processed using max projection on the LasX software (Leica) and taken for image analysis using FIJI [207].

2.17 Image analysis

Before images were analysed using a macro script, which largely automated the analysis process, some parameters such as “threshold level” and “particle size” needed to be optimized. A minimum of three randomly selected images was manually taken through the whole process to ensure that automation error would be kept to the minimum.

Nuclei were counted first by using a script used in FIJI (Fig. 2.8)


```

22 print("Number of files = " + allFiles.length);
23 print(resultsDir);
24
25     //nuclear counting loop
26     for(a=0; a<allFiles.length;a++){
27
28         //Open and analyse images
29
30         open(sourceDir + allFiles[a]);
31         run("Split Channels");
32         selectImage(allFiles[a] + " (red)");
33         close();
34         selectImage(allFiles[a] + " (green)");
35         close();
36
37         selectImage(allFiles[a] + " (blue)");
38
39         run("Set Measurements...", "area mean min integrated redirect=None decimal=3");
40         run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");
41
42         // Segmentation to find nuclei
43         run("Subtract Background...", "rolling=150");
44         run("Gaussian Blur...", "sigma=2");
45         setAutoThreshold("Li dark");
46         setOption("BlackBackground", false);
47         run("Convert to Mask");
48         run("Watershed");
49         run("Analyze Particles...", "size=100-1000 show=Masks summarize");
50
51         //save mask image
52         selectImage("Mask of " + allFiles[a] + " (blue)");
53         savePath=resultsDir + allFiles[a];
54         print("old name " + savePath);
55         savePath = replace(savePath,"\\.tif","_nuc.tif");
56         print("new name " + savePath);
57
58         saveAs("tiff",savePath);
59
60         close();
61
62 Kill_all_Images();
63
64     } //end of nuclear counting loop
65

```

Figure 2.8: Script used for counting nuclei of confocal images.

Before line 26 is the programming language that allows folder selection to select the images for analysis and designate an output folder. Line 26 starts a “For” loop where it will go through every picture in the folder before terminating. Line 30 opens the picture and Line 31 runs “Split Channels” where the RGB confocal image will be split into “red”, “green” and “blue” images. Since I am interested in counting the cells, which are stained using DAPI, the “blue” image was selected at line 37. Lines 39 and 40 are used to set measurement parameters, such as pixel area, and scale the picture in terms of

pixels, respectively.

Lines 43 to 49 are parameters that need “adjusting” to get accurate counts of nuclei. To enter correct parameters into these lines, a reference count is performed. To check if the script is counting correctly within a certain error of 10% (+4 if 40 cells are counted). The automated counts are compared to reference counts made by manually counting the cells of 3 randomly selected images.

Line 43 (“Subtract Background”) runs to reduce background levels to reduce false positives while line 44 (“Gaussian Blur”) runs to blur the image a little so that nucleolus will not form black patches within the nuclei that would impede analysis. This Gaussian blurring has a side effect as it also blurs the gaps in between nuclei in the epidermis so caution must be used. Line 45 runs an “auto-thresholding” algorithm “Li” that separates the positive signals from the negative ones. Line 45 also tells the software to invert background (“dark”). Positive signals above the threshold will be now regarded as “1” on a binary image and negative signals below the threshold will be now regarded as “0”. Line 46 informs the software that the image that it has analysed has a black background (Bright field images have a white background). Line 47 converts the image that has undergone processes described in lines 43-46 into binary images with only white (0) or black (1). When this happens, nuclei close to each other will be merged. Line 48 (“Watershed”) is included to separate merged nuclei, which would otherwise be counted as 1. After separating these nuclei, Line 49 “Analyze Particles”, analyses how many individual separate groups there are and outputs a numerical result (“summarize”). The size of particles may also be adjusted (“size=100-1000) to omit small particles that resulted from inaccurate “Watershed” procedure. A binary image

of nuclei that were counted was also output as a result (show=Masks). Lines 52 to 58 serve to save the output binary image into the output folder designated in program lines before line 26. Finally, the “For” loop is closed at line 64.

When the script outputs the results, the number of nuclei counted by the script is compared to the manually counted numbers to test if the script is adjusted accurately. If the automated count deviates too far ($>10\%$) from any of the 3 manually counted numbers, the parameters will be tweaked so that the automated count matches the manual counts within an error range of 10%.

```

76
77     for(a=0; a<allFiles.length;a++){
78
79         //Open and analyse images
80
81         open(sourceDir + allFiles[a]);
82         run("Split Channels");
83         selectImage(allFiles[a] + " (blue)");
84         close();
85         selectImage(allFiles[a] + " (red)");
86         close();
87
88         selectImage(allFiles[a] + " (green)");
89
90         run("Set Measurements...", "area mean min integrated redirect=None decimal=3");
91         run("Set Scale...", "distance=0 known=0 pixel=1 unit=pixel");
92
93
94         setThreshold(70, 255);
95         setOption("BlackBackground", false);
96         run("Convert to Mask");
97         run("Analyze Particles...", "size=1-Infinity show=Masks summarize");
98
99         selectImage("Mask of " + allFiles[a] + " (green)");
100        savePath=resultsDir + allFiles[a];
101        print("old name " + savePath);
102        savePath = replace(savePath, "\\_tif", "_dot.tif");
103        print("new name " + savePath);
104
105        saveAs("tiff", savePath);
106
107        close();
108
109    Kill_all_Images();
110
111    } //end of dot counting loop
112
113

```

Figure 2.9: Script used for counting area of signal of confocal images.

For quantifying Cx43 or other signals, stained by Alexa Fluorophore (AF)-488, the green image is selected after image splitting (Line 81 to 88). Instead of using an auto-thresholding method, a threshold is set to only include positive signals. This is done on the image referencing the split image to ensure only signals that can be observed are counted. After thresholding, the image is converted into a binary image (Line 96) and the image was analysed by including all particles sized 1 to infinity.

Differentiating epidermis from other parts of the skin requires a manual step by demarcating where the epidermis is. To do so, a region of interest (ROI) is drawn on the original colour image and saved on an “ROI Manager” tool in ImageJ. This saved ROI is then imprinted on the binary image and only the region within the ROI is analysed. This analysis will also output an image showing the analysed region. All output images and region of interest coordinates are saved for documentation. Measurements were only taken from the region of interest, in this case, the epidermis.

Figure 2.10: Images generated when running macros script. A) Image of a confocal image with ROI drawn to isolate only the epidermis for measurement. B) Binary image as a result of script automated process. C) Binary image of analysed nuclei in the demarcated zone.

2.18 RNA extraction

RNA extraction was performed according to the manufacturer's protocol using an RNeasy Fibrous Tissue Mini Kit (74704, Qiagen). Tissues previously stored in RNALater and stored at -80°C were thawed and weighed. No more than 30 mg of tissue was used for RNA extraction. Weight adjusted tissues were then immersed into 300 μL of Buffer RLT with an autoclaved metal ball added into the tube and loaded into a Tissue lyser for homogenization at 27 Hz for 3 min. Next, 10 μL of proteinase K in 590

μL of RNase-free water was added and incubated at 55°C for 10 min. Tissue debris was then removed by centrifugation at 12,000 x g for 3 min. The supernatant was collected into a new tube and approximately 450 μL of 96%-100% molecular grade ethanol was added. 700 μL of the sample was then transferred to the RNeasy Mini Column and centrifuged for 15 s at 9000 x g. This step was repeated until no sample was left. Next, 350 μL of Buffer RW1 was added and centrifuged for 15 s at 9000 x g. The previously prepared DNase1 solution by mixing 10 μL of DNase1 stock with 70 μL of Buffer RDD was added and incubated at room temperature for 15 min. After incubation, 350 μL of RW1 buffer was added and centrifuged at 9000 x g. This is followed by the addition of 500 μL of RPE buffer and this was also removed by centrifugation at 9000 x g for 15 s. Another 500 μL of RPE buffer was added and centrifuged for 2 min at 9000 x g to dry. The column was then transferred onto a new 1.5 mL tube for RNA elution by adding 30-50 μL of RNase-free water and spinning at 9000g for 1 min. Purified RNA was then measured using a NanoDrop One[®] (Thermo) to determine the concentration.

2.19 cDNA generation

To generate cDNA from purified RNA, the SuperScript VILO cDNA synthesis kit was used (11754-250, Invitrogen). Following the manufacturer's protocol, 1 μg of total RNA was added to a master mix containing 4 μL of the reaction mix, 2 μL of enzyme mix and water added to a final reaction volume of 20 μL. Tubes were gently mixed and incubated at 25°C for 10 min followed by 42°C for 1 h and the reaction was terminated by raising the temperature to 85°C for 5 min. All temperature incubations were performed using a thermal cycler. Tubes were then removed and either stored at -20°C or immediately used for qPCR.

2.20 Quantitative PCR

To perform qPCR, an Express SYBR GreenER kit (11780-200, Invitrogen) was used. Following the manufacturer's protocol, the reaction mix includes 10 μL of Express SYBR GreenER SuperMix, 0.5 μL of 10 μM of forward and reverse primers, 0.4 μL of 25 μM ROX reference dye and 1 μL of cDNA generated from the previous step. DEPC treated water was used to top up to a final reaction volume of 20 μL . Validated PCR sequences of TNF α , IL6, MMP-9 and GAPDH (Table 2.3) were obtained from Primerbank and purchased from IDT. Two technical replicates were prepared for all samples in qPCR compatible PCR tubes and loaded into the StepOnePlus™ Real-Time PCR Thermal Cycling Block (Applied Biosystems). All samples were run using a thermal cycling protocol of 3 phases. Phase 1 is 95°C for 10 min (polymerase enzyme activation) followed by phase 2 which consist of 40 cycles of 95°C for 15 s and 60°C for 1 min (thermal cycling). This is followed by phase 3 which consists of a final 95°C for 15 s and 60°C for 1 min (for melt curve analysis). Deviations in between technical replicates of >0.7 were excluded following value guides programmed into the qPCR machine (Applied Biosystems). After values were obtained, technical values were averaged. Relative fold gene expression was then calculated by using formula $2^{-\Delta\Delta\text{Ct}}$. First, the gene of interest Ct (cycle threshold) was normalised to their housekeeping gene Ct value by taking the difference between them (ΔCt). Next, the difference between an experimental sample and an experimental control (treated vs. untreated) is done ($\Delta\Delta\text{Ct}$). Finally, to find the expression fold change, values were brought up to the $2^{-\Delta\Delta\text{Ct}}$ and plotted on a graph for statistical analysis.

Table 2.5: Table of primer sequences used for quantitative PCR.

No.	Name	Sequence
1	Mus musculus tumour necrosis factor (TNF α)	Forward: CCCTCACACTCAGATCATCTTCT Reverse: GCTACGACGTGGGCTACAG
2	Mus musculus interleukin 6 (IL6)	Forward: TAGTCCTTCCTACCCCAATTTCC Reverse: TTGGTCCTTAGCCACTCCTTC
3	Mus musculus matrix metalloproteinase 9 (MMP-9)	Forward: CTGGACAGCCAGACACTAAAG Reverse: CTCGCGGCAAGTCTTCAGAG
4	Mus musculus glyceraldehyde-3-phosphate dehydrogenase (GAPDH)	Forward: AGGTCGGTGTGAACGGATTTG Reverse: TGTAGACCATGTAGTTGAGGTCA

2.21 Quantifying Cx43 in Neutrophils

2.21.1 Harvesting and processing bone marrow and blood

Neutrophils were isolated and purified using a neutrophil isolation kit for mice (130-097-658, Miltenyi Biotec). Following the manufacturer's instructions, all samples were kept on ice whenever possible. First, bone marrows and blood were harvested from five mice of each genotype (Cx43^{flx/flx}, Cx43^{flx/flx} LysMCre^{-/+} and Cx43^{flx/flx} LysMCre^{+/+}). Harvested bone marrows were flushed by inserting a 27-gauge needle into the femur bone using a wash buffer containing 1x PBS, pH 7.2, 0.5% bovine serum albumin and 2 nM EDTA. After flushing, cells were immediately counted using ethylene blue on a haemocytometer and centrifuged at 300 x g for 10 min. The supernatant was removed completely, and the cell pellet was resuspended in 200 μ L of wash buffer. Next, 50 μ L of Neutrophil Biotin-Antibody Cocktail provided in the kit was added for every 5 x 10⁷ cells. The cell suspension was then mixed and incubated at 2 °C - 8 °C for 10 min. Next, the cells were washed by adding 5 mL– 10 mL of buffer for every 5 x 10⁷ cells and centrifuged at 300 x g for 10 min. All supernatant was removed, and the cell pellet was resuspended in 400 μ L of buffer for every 5 x 10⁷ cells. Next, 100 μ L of Anti-Biotin

MicroBeads provided in the kit was added for every 5×10^7 cells and incubated at 2°C - 8°C for 10 min. Cells were washed again by adding 5 mL– 10 mL of buffer for every 5×10^7 cells and centrifuged at $300 \times g$ for 10 min. Finally, all supernatant was removed completely, and the cell pellet was resuspended up to 10^8 cells in 500 μL of wash buffer.

2.21.2 Neutrophil purification

For optimal purity and recovery of neutrophils from bone marrow and blood, an LS Column (130-042-401, Miltenyi Biotec) was used. The LS column was loaded onto a MidiMACS™ Separator (130-042-302, Miltenyi Biotec) and magnetically attached to a MACS MultiStand (# 130-042-303, Miltenyi Biotec). Next, the column was primed by adding 3 mL of buffer followed by adding the cell suspension into the column. The flow-through containing unlabelled cells was collected and this would contain an enriched population of neutrophils. The column was then washed three times with 3 mL of buffer and combined with the flow-through.

2.21.3 Neutrophil staining

For imaging Cx43 on neutrophils, 3 coverslips per mouse were prepared. To stain the neutrophils, coverslips were coated with 0.1 mg/mL of polylysine solution. First, the coverslips were placed into a 24-well plate. Next 350 μL of 100 % ethanol was added to sterilize for 1 h at room temperature. Subsequently, the 100 % ethanol was removed and washed with 350 μL of 1x PBS. After 1x PBS was removed, 350 μL of 0.1 mg/mL polylysine solution was added and incubated for 1 h at room temperature. Coverslips were also exposed to ultraviolet light during this incubation for sterilisation. Finally, the polylysine solution was removed and the coverslip was

allowed to dry.

After coverslips were coated in the 24-well plate and dried, 350 μ L of cell suspension was added into the wells and incubated for 1 h to allow for neutrophils to adhere. Next, wells were aspirated, and samples were then fixed by adding 350 μ L of 4 % paraformaldehyde at room temperature for 15 min. Samples were then washed by removing the 4% PFA and 350 μ L of 1x PBS was added and incubated for 5 min. This was followed by 350 μ L of permeabilising buffer containing 0.1% Triton-X 100 in 1x PBS for 10 min. Next, the permeabilising buffer was removed and blocking buffer containing 1% BSA in 1x PBS was added and incubated for 1 h at room temperature. After removing the blocking buffer, 350 μ L of primary antibody solution containing rat anti-mouse Ly6g (127602, Biolegend) at 1:500 and rabbit anti-mouse Cx43 (C6219, Sigma) at 1:1000 was added and incubated overnight at 4°C. After overnight incubation, wells were washed two times of 350 μ L of 1x PBS for 5 min. After removing the 1x PBS, 350 μ L of secondary antibody solution containing goat anti-rabbit AF488 (A11008, Thermo) at 1:500 and goat anti-rat AF568 (A11077, Thermo) at 1:500 was added and incubated for 1 h. After incubation, wells were washed two times of 350 μ L of 1x PBS for 5 min. After removing the 1x PBS, 350 μ L of DAPI solution was added at 1:10000 dilution and incubated for 10 min. After incubation, the well was first washed with 350 μ L PBST for 5 min followed by 350 μ L 1x PBS for 5 min. Coverslips were taken out of the well and the underside of the coverslip was dried. Cover-slipping was done by applying one drop of Citifluor™ onto the glass slide and inverting the coverslip on the droplet. The coverslip was allowed to rest for 5 – 10 min before the coverslip was secured with nail varnish on the sides.

2.21.4 Imaging and analysing Cx43 in neutrophils

After cover-slipping, three fields of view per coverslip were taken using Leica SP8 upright confocal microscope (Leica, Germany) using a 40x objective (506358, 1.3NA, oil, PL, APO, Leica). Confocal images were taken in 1024 by 1024 format with 8-bit depth. A total of nine fields of view were imaged for each mouse. All cells that were positive for Ly6g (~100 cells per sample) were individually analysed using ImageJ for Cx43 pixel area and were averaged to generate a single data point.

2.22 Statistics

All statistics were performed on the GraphPad Prism® 6 software. When comparing two independent, non-normalised groups, Mann-Whitney U-test was used to determine the level of significance where distribution was not normal or undetermined. When comparing two independent normalised groups, Wilcoxon signed-rank test was used. To compare in between more than two independent groups, the non-parametric Kruskal-Wallis test followed by Dunn's post hoc test was used. To compare between groups with 2 variables (score and time), a two-way ANOVA followed by Bonferroni's multiple comparisons test was used. Data that was converted for Kaplan Meier analysis was analysed using the Log-rank (Mantel-Cox) test. A p-value of <0.05 was considered significant. All error bars denoted the standard deviation of the mean except for 2-way ANOVA, which is denoted as the standard error of the mean.

Chapter 3. Characterisation of an ischaemia reperfusion model for the study of early events in pressure ulcer formation in mouse skin

3.1. Introduction

There is a scarcity of publications addressing the early biological processes of PU formation. Studying early events that cause PU formation in man would require biopsies of Human Stage 1 PU. However, current guidelines would prevent taking a biopsy because it would cause more damage to the Stage 1 PU. Any biopsies would have to be obtained from cadavers. Animal models are required for the study of early events in PU formation.

3.1.1. Animal models of pressure ulcers

Several mouse models have been designed to emulate a PU based on ischaemia reperfusion. These models have mainly centred on occluding blood flow by the application of paired magnets to the skin, causing local ischaemia. Removal of the magnets allows reperfusion of the blood, which results in a reperfusion injury (Table 3.1). The models can be divided into the implanted magnet models or the magnet pinch models.

Implanted magnet models involve the surgical implantation of steel plates or magnets under the skin. Ischaemia occurs when an external magnet is applied [208, 209]. Magnet pinch models involve the tenting of dorsal skin and applying magnets on either side, resulting in a “pinch”. The pinch causes ischaemia, without the need for any invasive procedure [204, 210]. Early publications used long periods of ischaemia and

were examined using a human PU grading system [204]. By day 5, the ischaemia durations of 12 hours resulted in an ulcer stage 2.4 and peaked at an ulcer stage of 3.6 by day 10, with the loss of epidermis, dermis and subcutaneous tissue [204]. Table 3.1 tabulates the ischaemia and reperfusion durations for implanted magnet models and magnet pinch models in different animal models. Tabulation of these publications revealed that the histology of ischaemia reperfusion injury was not very well documented or described. The use of shorter ischaemia durations, in conjunction with stronger magnets, was not explored in mice. This novel combination may allow for the quick generation of a PU.

There are some limitations in using mice to study PU. For example, Stadler et al. created and characterised the magnet pinch model using a scoring matrix meant for identifying human PU. Important differences between mouse and human skin include the presence of the panniculus carnosus (PC) in rodents, which may be mistaken for underlying skeletal muscle [211]. This unique presence of PC in mice and the lack of bone located directly below the skin present a problem in unifying the more severe ulcer stages (Fig. 3.1)

Figure 3.1: Figure showing the difference between a theoretic Stage 4 PU in a mouse and a human Stage 4 PU.

Mice are also loose skinned animals that often heal by wound contraction. This happens very quickly, and any injury would rapidly heal and not develop into a chronic wound. Despite mismatches of later PU stages of humans to mice, the magnet pinch mouse model is still suitable for generating an injury like a Stage 1 PU, which could shed light on human Stage 1 PU dynamics.

Table 3.1: Table of publications using magnets to create I/R injuries mimicking pressure ulcers.

Year	Papers	Ischaemia reperfusion (I/R) timings	Magnet procedure	Animal model	Magnet strength	Histology
1993	Fukumura et al. [212]	6 weeks of ischaemia	Steel plate under skin, external magnet	Minipigs	10, 20 mm Hg	present but unexplained
2000	Peirce et al. [208]	I/R = 2 h, 0.5 h *5 times + 11.5 h for 1,2,3,4,5 days I/R = 1 h, 0.5 h *5 + 16.5 h for 5 days I/R = 1 h, 0.5h *10 + 9 h for 5 days	Steel plate under skin, external magnet	Sprague–Dawley rats	1250 Gauss, 50 mm Hg	present but some observations unexplained

		I/R = 10 h, 0 or 2, 0.5*5 for 1 day				
2001	Peirce et al. [213]	I/R = 2 h, 0.5 h * 5 times + 12 h + 2 h, 0.5 h * 5, +12 h or +31 h	Steel plate under skin, external magnet	Sprague–Dawley rats	1250 Gauss, 50 mm Hg	only leukocyte description
2004	Lanzafame et al. [210]	I/R = 12 h, 12 h repeated for 3 days	Ceramic magnets external pinch	C57Bl6 mice	1000 Gauss	Absent
2004	Stadler et al. [204]	I/R = 12 h, 12 h repeated for 3 days	Ceramic magnets external pinch	BALB/c mice	1000 Gauss	Present but inaccurate and not extensive
2006	Şener et al. [214]	I/R = 2, 0.5 * 5 + 11.5 for 3 days	Steel plate under skin, external magnet	Wistar Rats	1250 Gauss	Present but not properly explained
2006	Şener et al. [215]	I/R = 2, 0.5 * 5 + 11.5 for 5 days	Steel plate under skin, external magnet	Wistar Rats	not mentioned but cited Pierce	Present but not indicative
2007	Sisco et al. [209]	I/R = 2, 6 h or 24 h x 2	Magnet under skin, external magnet	Sprague–Dawley rats	82 mm Hg	Present but only aimed in subcutaneous leukocyte description
2008	Saito et al. [216]	I/R = 12, 12 for 3 days	Ceramic magnets external pinch	C57Bl/6, MCP-1-/- mice	1000 Gauss	Present but describing edge of insult and not informative
2008	Hashimoto et al. [217]	I/R = 50 mmHg 4 h or 100 mmHg 2h, 3 h, 4 h; 12 h, 1 day, 3 day, or 1 week	Neodymium magnet under skin, external magnet	Wistar Rats	2,100 Gauss, 50- or 100-mm Hg	Present but mainly describing muscle layers
2009	Wassermann et al. [218]	I/R = 2 h, 1 h for 1, 4, 6, 8, 10 cycles	Steel plate under skin, external neodymium nickel magnet	BALB/c nu/nu nude mice	1.29 T	Present but not extensive
2011	Kawamata et al. [205]	I/R = 4, 20 x 5 days harvest 2d, 4, 8, 12, 16, 20 I/R = 4, 20 x 2- or 4-days harvest 1 day after	Gold-plated magnet under skin, external magnet	Wistar rats	100 mm Hg	Present includes dermis, skin muscle. Lacking other descriptions
2011	Tong et al. [219]	I/R = 16 + 1, 2, 3, 4, 7, 14, 21, 42, 84 days	Magnet pinch	WAG/Rij Hsd rats	2,300 Gauss	Present for 3, 7, 14, 21 for CD68 (monocyte/macrophage), 7, 14, 21, 42 (endothelial

						angiogenesis) for CD34 IHC
2011	Ed la Garza- Rodea et al. [220]	I/R = 12 + 8 h, 1 ,2,4,8,13,20 days	Magnet pinch	SKH1-hr, NOD- LtSz- scid/scid/J (NOD/SCI D) mice	75kPa (562mm Hg)	Present for 8,1,2,4,8,13,20 mainly describing occurrences at panniculus carnosus
2013	Nguyen- Tu et al. [221]	I/R = 4 h + 24 h	Metal plate under skin, external magnet	C57Bl/6 mice	11kPa	Absent
2015	Strong et al. [222]	I/R = 12 h+ 12 h, 2 times	Magnet pinch	C57Bl/6 mice	not mentione d	Present with PBS and mainly measured for quantity not quality
2015	Kawamata et al. [223]	I/R = 4 h + 20 h, 5 times	Gold magnet under skin, external neodymium magnet	Wistar rats	50, 60, 70 mmHg	Present but not informative
2015	Strong et al. [224]	I/R = 12 h +12 h, 2 or 3 I/R cycles	Magnet pinch	C57BL/6 mice	0.183N	Present with mainly measured for quantity not quality
2015	Kurose et al. [225]	I/R = 4 h + 12 h, 1 day or 3 days	Neodymium magnets under the skin and over	Wistar rats	100 mm Hg	Present but only describing thickness and indications of necrosis and inflammation
2015	Zheng et al. [226]	I/R = 12 h+12 h x 2 days	Ceramic magnets pinch	Sprague Dawley rats and BALB/c	3500 gausses	Present but with little description
2015	Swisher et al. [227]	I/R = 1 h or 3 h, 1, 2, 3 and 9 days	round, flat magnets	Sprague Dawley rats	1000 gauss	Present describing epidermis and inflammation
2017	Schivo et al. [228]	I/R = 1.5 h + 1 h harvest	Neodymium magnet pinch on ear	NZ white rabbits	48.66 mm Hg cm ⁻²	Absent

3.1.2 Human pressure ulcer features

In a publication on human Stage 1 PU, histological profiles of the wounds were described in detail [28]. These included the presence of epidermal blistering, necrosis of epidermis, and follicular damage. In a Stage 2 PU, the epidermis was lost, and collagen fibres were fewer and thicker compared to normal tissue. Tissue adjacent to the injury contained dense collagen fibres and adipocytes [28].

In more severe PU, a publication by Sutcliffe et al. reported that the gap junction protein Cx43 was upregulated in the epidermis and dermis in the tissue 1mm adjacent to PU [158]. Cx43 upregulation in the epidermis and dermis could play a role in the spreading of death signals through the bystander effect causing the PU to progress to more advanced stages [184, 189].

Wound edges of other chronic wound types such as diabetic foot ulcers and venous leg ulcers also have Cx43 upregulation in the epidermis and dermis [158]. Among the three ulcers, venous leg ulcers and PU are similar as they are progressive. Compared to the 4 stages of PU, there are 6 classifications or stages of venous disease. Venous diseases are classified according to CEAP (Clinical, Etiological, Anatomical, Pathophysiological) [229]. They are: C0 (no visible venous disease), C1 (spider veins), C2 (varicose veins), C3 (oedema), C4 (lipodermatosclerosis), C5 (healed venous leg ulcer) and C6 (active venous leg ulcer). It was reported that Cx43 protein was upregulated in the epidermis of skin progressing from C0 to C2 to C4 to C6 [230]. This may also be the case for the progression of a Stage 1 PU to a Stage 4 PU.

3.1.3 IL-6, TNF α and MMP-9 as an indication of PU severity

In PUs, cytokines such as IL6, TNF α , IL1 β alongside MMP-2 and MMP-9 are up-regulated [225, 231]. Inflammation may start early after ischaemia reperfusion injury as the insulted cells release damage-associated molecular patterns (DAMP) to recruit neutrophils to the area [18]. Little is known about what these neutrophils do in the context of PU formation but in a publication on cystic fibrosis, it was shown that neutrophils release ROS and elastases as part of the innate host defence response in sterile inflammation[232]. In the case of a sterile injury such as ischaemia reperfusion, neutrophils have no infection to fight off and they do harm to surrounding tissue [233]. As the PUs progress to Stage 2, inflammation increases and open wounds are now susceptible to bacterial infection such as by *Staphylococcus aureus* and *Pseudomonas aeruginosa* [234]. Infection complicates the wound and may aid in its progression and cause tissue necrosis.

The study of early processes of a Stage 1 PU requires the evaluation of a suitable ischaemia reperfusion duration. This optimised ischaemia reperfusion duration should result in an experimental PU which does not result in an open wound. The aim in this part of the project was to develop and characterise a mouse model suitable for studying the early processes that may be involved in PU progression. Currently, there are two predominant I/R models (magnet pinch model and magnet under the skin model) used in publications studying experimental pressure ulcers tabulated in Table 3.1. As such, there is a need to test the suitability of the models in studying the early process of PU formation. Cx43 will be the protein of interest as it is known to be involved in ischaemia reperfusion, inflammation, wound healing and has been reported to be upregulated at the wound edges of human PUs [158, 230, 235].

3.2. Results

3.2.1 The magnet pinch model is less variable than the magnet implantation model

To determine which mouse model is more suitable for the study of early PU formation, the magnet implantation model and the magnet pinch model were compared. The duration of 1.5 h of ischaemia was selected based on the work of a previous PhD student and the observation that 3 h of ischaemia using a 1000 gauss magnet in rats resulted in a more severe insult compared to 1 h of ischaemia [227]. After 1.5 h of ischaemia and 24 h of reperfusion (1.5 h(I) / 24 h(R)), the implantation model resulted in variable macroscopic phenotypes. Some of the insulted regions exhibited full-thickness skin loss that exposed the magnet (Fig. 3.2d, f). In one case, the translucent skin was attached by fibrous tissue to the gold-plated magnet (Fig. 3.2e). Other insulted regions exhibited little to no outward macroscopic phenotype (Fig. 3.2b, c). The magnet pinch model results were less variable. The insulted skin showed some discolouration with little variation 24 h post ischaemia (Fig 3.2g-l). There was also no difference between the two sides (Fig. 3.2 g, h, i, vs j, k, l). To test my hypothesis of Cx43 playing a role in the initial processes of PU progression, the model needs to result in an injury reflecting a Stage 1 PU. Since the implanted magnet model was variable and some resulted in a wound, the magnet pinch model was selected.

Figure 3.2: The magnet pinch model shows less variability and is reproducible after 1.5 h(I) / 24 h(R). a-f) Variable phenotypes were observed in the magnet implantation model. g-i) The magnet pinch model shows no epidermal breakdown and is less variable. White dotted line demarcates insult site. (N=6, 1 skin image per mouse for magnet implantation model. Pinch model N=3, 2 skin images per mouse. Scale bar = 5 mm.)

3.2.2 1.5 h of ischaemia was the best to study early events in pressure ulcer formation

The duration of ischaemia was optimised to find the maximum duration without progressing into an open wound. Durations of 1.5 h, 2 h, 2.5 h and 3 h of ischaemia were tested. After 1.5 h(I) / 24 h(R), a blanched skin appearance was observed with little variation between mice (Fig. 3.3a-c). As the duration of ischaemia increased, macroscopic severity became more evident with 2 h of ischaemia showing mild redness, which increased with 3 h of ischaemia resulting in non-blanchable erythema (Fig. 3.3d-l) and elevation of the outer skin layers (Fig. 3.3l). In addition, variation of macroscopic severity between mice was observed when 2 h(I) / 24 h(R) or 2.5 h(I) / 24 h(R) was used. This suggests that ischaemia duration of 1.5 h was the maximum duration for the reliable formation of a Stage 1 PU. Increasing ischaemia duration to 2

h, 2.5 h and 3 h would result in a more severe I/R injury with 2 h and 2.5 h resulting in variable insulted skin severity.

Figure 3.3: Ischemia above 1.5 h(I) results in a severe phenotype. a-c) Representative macroscopic pictures of skin that underwent 1.5 h(I) / 24 h(R) showing a blanched phenotype but no redness or skin breakage. d-f) Skin that underwent 2 h(I) / 24 h(R) showing little to mild redness. g-i) Skin that underwent 2.5 h(I) / 24 h(R) showing mild to deeper redness. j-l) Skin underwent 3 h(I) / 24 h(R) showing redness with epidermis peeling off. N=3, each image is taken from different mice. Scale = 5 mm

3.2.3 The insult site did not progress into a more serious injury after 1 cycle of 1.5 h of ischaemia.

Ischaemia reperfusion of 1.5 h(I) / 24(R) did not result in any obvious macroscopic phenotype. The possibility that in time it might progress into a more severe injury was investigated. The skin showed little signs of progression into a more severe injury over 8 days (Fig. 3.4a). From day 1, the skin appeared blanched with no changes progressing into day 2. On day 4, a small reddish spot appeared but disappeared by day 6. By day 8, the skin was still blanched. H&E staining showed the epidermis was intact through to 8 days (Fig. 3.4 c-f). For in-depth characterisation of histological changes, higher magnification images were acquired and described in the following sections (Fig 3.5-3.8).

Figure 3.4: Injury caused by 1.5 h of ischaemia did not progress into a more serious injury and did not result in epidermal breakage. a) Panel of representative macroscopic pictures of injured skin from day 1 to day 8. Picture shows non-progression of I/R injury with persisting blanched phenotype. b) Normal skin histology showing intact epidermis. c) Skin at 4 h post ischaemia showed intact epidermis d) Skin at 24 h post ischaemia showed intact epidermis. e) Epidermis at 4 days post ischaemia showed thicker but intact epidermis. f) Skin at 8 days post ischaemia showed intact epidermis similar to normal. (N=6, Scale = 200 μ m)

3.2.4 Histological characterisation of 1.5 h of ischaemia with 4 h reperfusion

Characterisation of skin that underwent 1.5 h of ischaemia and 4 h reperfusion was performed on H&E images from normal skin, skin from the surrounding tissue of the insult (non-insulted skin continuous with the insulted skin), the edge of insult (boundary of insult), and the centre of insult. At 1.5 h(I) / 4 h(R), keratinocytes showed condensed nuclei at the edge and centre of the insult (Fig. 3.5d, e). No obvious changes were noted in the hair follicles at 4 h. However, slightly condensed sebaceous gland nuclei were observed in the centre of the insult (Fig. 3.5f-i). Leukocytes were observed in blood vessels in the surrounding tissue and edge of the insulted skin (Fig. 3.5k, l). The insult centre had few blood vessels and leukocytes had extravasated (Fig. 3.5m). Dermal fibroblasts were observed to have lost their distinctive flattened star-like morphology at the edge of insult and were absent from the centre of the insult (Fig. 3.5o-q). The PC showed some degradation at the edge and centre of insult, with a loss of muscle fibres (Fig. 3.5t-u). There were no differences between normal skin and the skin in the surrounding tissue (Fig. 3.5b-r, c-s).

Figure 3.5: Histological observations of skin at 1.5 h(I) / 4 h(R). a) Insult orientation image of skin section. Coloured squares show where pictures were acquired: from the surrounding tissue (green), the edge of injury (orange) and the centre of injury (red). The red line demarcates the extent of the injury area. b-u) Representative histological images. b-e) epidermal nuclei appear to condense and this is more pronounced towards the centre of injury. f-i) Hair follicles in the centre show no obvious injury at 4 h. j-m) Leukocytes were observed in vessels in the surrounding tissue with leukocytes extravasating in the edge and centre of the injury. n-q) The dermis shows slight oedema as indicated by the expansion of the spaces in between ECM components. There was a loss of fibroblasts. r-u) Damage to the PC is also suggested at the edge and centre of injury with fewer muscle cells. Scale bar = 50 μ m. N=3.

3.2.5 Histological characterisation of 1.5 h of ischaemia with 24 h reperfusion

At 1.5 h(I) / 24 h(R), “spongiosis” or intercellular oedema was observed in the epidermis at the edge and the centre of the insult as indicated by the presence of gaps in between keratinocytes (Fig. 3.6d, e). Pockets of inflammatory cells were seen forming under the epidermis at the edge of the insult (Fig. 3.6d). Hair follicles showed no obvious signs of change except for the loss of sebaceous gland nuclei in the centre of the insult (Fig 3.6f-i). Leukocyte infiltration was observed at the edge and centre of insult (Fig 3.6l, m). The dermis at the edge of the insult had leukocytes amongst the fibroblasts. The centre of the insult was devoid of fibroblasts but had large numbers of leukocytes (Fig 3.6p, q). The PC at the edge and centre of insult was also observed to be damaged and muscle fibres were thinner than normal (Fig 3.6r-u).

Figure 3.6: Histological observations of skin at 1.5 h(I) / 24 h(R). a) Overall snapshot of skin section. Coloured squares show where pictures are taken from the surrounding tissue (green), the edge of injury (orange) and the centre of injury (red) and red line above demarcates injury area. b-u) Panel of pictures showing key differences. b-e) Neutrophil pockets were observed at the edge of the epidermis. Spongiosis was observed in both the edge and centre of injury. f-i) Hair follicles in the centre show no obvious injury at 24 h except the loss of nuclei in sebaceous glands. j-m) Leukocytes were observed at the edge and centre of the injury. n-q) Dermis shows loss of fibroblasts with leukocytes present in the injury area. r-u) Damage to the PC is also observed at the edge and centre of injury showing loss of muscle cells and muscle mass. Scale bar = 50 μ m. N=3.

3.2.6 Histological characterisation of 1.5 h of ischaemia 4 d after reperfusion

By the 4th day, epidermal spongiosis at the edge was reduced, while the centre exhibited more spongiosis (Fig 3.7d, e). Epidermal thickness was increased compared to both the surrounding tissue and normal skin (Fig 3.7d, e). Spongiosis was seen in hair follicles at the centre (Fig. 3.7i). Leukocytes were still in the centre of the insult (Fig 3.7m). Fibroblasts repopulated the previously injured dermis (Fig 3.7q). Healing of the PC was also observed (Fig 3.7t, u).

Figure 3.7: Histological observations of skin at 1.5 h(I) / 4 d(R). a) Overall snapshot of skin section. Coloured squares show where pictures are taken from surrounding tissue (green), edge of injury (orange) and centre of injury (red) and red line above demarcates injury area. b-u) Panel of pictures showing key differences. b-e) Spongiosis was observed in both the edge and centre of injury. Epidermis is hyperthickened. f-i) Hair follicles in the centre show signs of spongiosis. j-m) Leukocytes were still observed at the edge and centre of the injury. n-q) Dermis shows an increase in fibroblast with leukocytes still present in the injury area. r-u) Regeneration of PC is observed at edge and centre of injury. Scale bar = 50 μ m. N=3.

3.2.7 Histological characterisation of 1.5 h of ischaemia 8 d after reperfusion

By the 8th-day post insult, the epidermis had less spongiosis and the thickness started to reduce compared to 4 days post-injury (Fig 3.8d, e). There were no observable differences in the hair follicles (Fig 3.8f-i). Vessels at the edge and the centre of the insult retained the presence of leukocytes in the lower dermal regions (Fig 3.8l, m). The dermis was populated with more fibroblasts and was still recovering at the centre of insult (Fig 3.8p, q). Muscle fibres at the edge and centre of the insult had not completely recovered indicated by nuclei not repositioning to the edge of the muscle fibre (Fig 3.8r-u).

Figure 3.8: Histological observations of skin at 1.5 h(I) / 8 d(R). a) Overall snapshot of skin section. Coloured squares show where pictures are taken from the surrounding tissue (green), edge of injury (orange) and centre of injury (red) and red line above demarcates injury area. b-u) Panel of pictures showing key differences if any. b-e) Less spongiosis was observed in both the edge and centre of injury when compared to 4 d(R). Epidermis is less hyper-thickened. f-i) Hair follicles look normal. j-m) Leukocytes were still observed in the vessel at the edge and at the centre of the injury. n-q) Dermis shows fibroblast reappearing. r-u) Regeneration of PC is still on-going at edge and centre of injury shown by muscle with central nuclei. Scale bar = 50 μ m. N=3.

3.2.8 Evidence of vascular damage after 1.5 h of ischaemia / 24 h of reperfusion

In-depth histological characterisation revealed that blood vessels seemed to be non-functional in the centre of the insult based on the lack of defined vessels in histological sections (Fig. 3.5m and 3.6m). To investigate further and map out the microvasculature in the skin, FITC-BSA was used to mark out the blood vessels. There were two vascular abnormalities observed. First, at the edge of insult, FITC-BSA leaked out of the blood vessels (Fig 3.9d, orange dotted box) when compared to the normal skin that has FITC-BSA contained within the blood vessels (Fig 3.9b). This suggests that there was vessel hyper-permeability. In the centre of insult, there was a lack of well-defined blood vessels filled with FITC-BSA when compared to the normal skin (Fig. 3.9d, red dotted box) which suggest that there was a lack of functioning vessels.

Figure 3.9: Two vascular abnormalities observed at 1.5 h(I), 24 h(R). Representative confocal images showing FITC-BSA in skin. a) Confocal image of a single view showing FITC-BSA (green) contained in the vessel within the normal skin. b) Confocal montage showing FITC-BSA in vessels in the skin over a larger area of normal skin. c) Confocal picture of a single view showing FITC-BSA that has leaked out into the skin at 1.5 h(I), 24 h(R). d) Confocal montage showing leaked FITC-BSA at the edge of insult (orange dotted box) and fewer blood vessels at the centre of insult (red dotted box) indicated by the lack of FITC-BSA. Scale = 50 μ m and 1 mm. N=3.

3.2.9 Necroinflammatory debris observed after 1.5 h of ischaemia / 24 h of reperfusion consisted of mainly neutrophils accumulating under the epidermis

Necroinflammatory debris (NID) was observed in regions of the epidermis (Fig. 3.6d, Fig. 3.9c). When compared to the normal skin, it seems that Cx43 expression has been lost in the epidermis above NID while some Cx43 expression can be detected in the NID (Fig 3.10b).

Figure 3.10: Necroinflammatory mass consists of mainly neutrophils at the epidermis. a) Confocal image showing normal skin stained with Cx43 (green) and Ly6g (red). No neutrophils were observed. b) Confocal image showing insulted skin with Ly6g +ve neutrophil masses gathered under the epidermis. Scale bar = 50 μm. N=3.

3.2.10 Connexin43 protein levels in the epidermis increase after 1.5 h ischaemia 4 h and 24 h reperfusion

Histological changes in the epidermis were observed at 1.5 h(I) / 4 h(R) and 24 h(R) which prompted the investigation of Cx43 protein levels through IHC staining (Fig. 3.11, 3.12, in red). A significant increase (1.89x increase) in epidermal Cx43 protein levels in the insulted region was observed as early as at 4 h ($p < 0.05$, *, Fig. 3.11c) when compared to Cx43 in the normal epidermis (Fig. 3.12c). These levels continued to increase (3.23x increase) after 1.5 h(I) / 24 h(R) ($p < 0.05$, *).

Figure 3.11: Cx43 protein levels increased 4h post 1.5h of ischaemia. a) Representative confocal image showing normal Cx43 protein levels (red) in the epidermis and is b) increased 4h post 1.5h ischaemia. c) Graph reporting quantified protein levels with insulted skin being significantly higher than normal. (n=3, $p < 0.05$, Wilcoxon signed-rank test) Scale bar = 50µm.

Figure 3.12: Cx43 protein levels are increased 24 h post 1.5 h of ischaemia. a) Representative confocal image showing normal Cx43 protein levels in the epidermis and is b) increased 24 h post 1.5 h ischaemia. c) Graph of Cx43 protein levels with insulted skin being significantly higher. (n=3 for normal skin and n=6 skin samples from 3 mice, $p < 0.05$, Mann-Whitney U-test) all data plotted with mean (SD) Scale bar = 10µm.

3.2.11 Double cycle pinch model 1.5 h(I) / 1.5 h(R) / 1.5 h(I) / 11 d(R) results in an experimental Stage 2 PU.

An extension to the established single cycle magnet pinch mice model was required to allow testing of treatments to prevent the progression of Stage 1 to Stage 2 PU. The effect of an additional cycle of 1.5 h(I) after 1.5 h(R) and 24 h(R) was chosen to establish the duration of reperfusion that was required to generate a stage 2 PU. By using an additional cycle of 1.5 h(I) after 1.5 h(I) / 1.5 h(R), the injury progressed to an open wound at day 3. After which, the wounds began to heal, and the process was completed by day 11 (Fig 3.13).

Figure 3.13: Double cycle of 1.5 h(I) / 1.5 h(R) / 1.5 h(I) resulted in ulcer progression with peak injury at day 3. Panel consists of images of left and right insulted skin over 12 days. Injury peaked at day 3 and started healing. Most of the healing is completed by day 11. N=2. Scale bar = 5 mm

3.2.12 Double cycle pinch model 1.5 h(I) / 24 h(R) / 1.5 h(I) / 11 d(R) results in an experimental Stage 2 PU.

When an additional cycle of 1.5 h(I) after 1.5 h(I) and 24 h(R) was used, the insult resulted in a more severe phenotype (Fig 3.14, Day 3) compared to 1.5 h reperfusion cycle (Fig. 3.13, Day 3). Similar to double cycle of 1.5 h(I) / 1.5 h(R) / 1.5 h(I) (Fig. 3.13), the injury also peaked on day 3 and started healing (Fig. 3.14). Double cycle of 1.5 h(I) / 24h(R) / 1.5 h(I) also completed healing at day 11 with some scar tissue (Fig. 3.14).

Figure 3.14: Double cycle of 1.5 h(I) / 24 h(R) / 1.5 h(I) resulted in ulcer progression with peak injury at day 3. Panel consists images of left and right insulted skin over 11 days. Injury peaked at day 3 and started healing. Most of the healing is completed by day 11. N=2. Scale bar = 5 mm

3.2.13 Histological differences of a single pinch (1.5 h(I) / 4 h(R)) compared to a double pinch (1.5 h(I) / 4 h(R) / 1.5 h(I) / 24 h(R))

Reperfusion durations of 1.5 h and 24 h resulted in Stage 2 PU which suggest that any reperfusion durations between 1.5 h and 24 h should result in a Stage 2 PU (Fig. 3.13, 3.14). The 4 h(R) reperfusion duration was chosen as it will be valuable to know what state of the skin is in right before the second pinch (Fig. 3.5 and Fig. 3.11).

1.5 h ischaemia was performed to an already insulted skin which underwent 1.5 h(I) / 4h(R), and spongiosis was observed at the edge of the injury (Fig 3.15d). However, the epidermis was no longer present in the centre of the injury and was replaced by necroinflammatory debris (Fig. 3.15e). Hair follicles at the edge showed no major difference but sebaceous glands showed some nuclei loss (Fig. 3.15h). There was a lack of hair follicles in the centre of injury and any remaining ones were surrounded by leukocytes (Fig. 3.15i). Leukocytes were observed in blood vessels in skin far away from the insult (surrounding tissue, Fig. 3.15k). Leukocytes were also found to be at the edge and centre of injury (Fig. 3.15l, m). There was a lack of dermal fibroblast nuclei in both the edge of injury and the centre of the insult (Fig. 3.15p, q). The PC also showed considerable damage at the edge of injury and loss of whole muscle fibres were observed in the central insulted region with the loss of muscle nuclei (Fig. 3.15t, u). This suggests that a double cycle was able to generate a more severe injury similar to a Stage 1 PU transitioning into a Stage 2 PU.

Figure 3.15: Histological observations of skin at 1.5 h(I) / 4 h(R) / 1.5 h(I) / 24 h(R). a) Overall snapshot of skin section. Coloured squares show where pictures are taken from the surrounding tissue (green), edge of injury (orange) and centre of injury (red) and red line above demarcates injury area. b-e) Spongiosis was observed at the edge of injury and necroinflammatory debris was observed in the centre. f-i) Hair follicles in the centre show complete loss of hair follicular cells at 24 h(R). j-m) Neutrophils were observed at the edge and centre of the injury. n-q) Dermis at the edge and centre of the insult lost fibroblasts with neutrophils present in the injury area. r-u) Damage to the PC is observed at edge and centre of insult showing loss of muscle nuclei and mass. Scale bar = 50 μm.

3.2.14 Cytokine and MMP-9 mRNA validation of a single pinch 1.5 h(I) / 24 h(R)
compared to a double pinch 1.5 h(I) / 4 h(R) / 1.5 h(I) / 24 h(R)

Histology revealed that the double cycle was able to generate a more serious injury. An increase in cytokines interleukin-6 and tumour necrosis factor-alpha mRNA level would mean a more inflamed state of the tissue. To determine if the double pinch resulted in a higher inflammatory state, mRNA of interleukin-6 (IL6) and tumour necrosis factor-alpha (TNF α) were measured and compared to their controls before comparing to the single pinch. Matrix metalloproteinase-9 was also measured as MMP-9 protein levels are elevated in human non-healing PU [231]. In a single pinch, mRNA of TNF α , IL6 and MMP-9 were not increased (n=5, p>0.05, ns) when compared to normal skin (Fig. 3.16a-c). When a double pinch was applied, all three were significantly increased (p<0.05, *) compared to the single pinch (Fig. 3.16a-f).

Figure 3.16: Double pinch shows higher mRNA levels measured by qPCR of known cytokine markers and MMP-9 than single pinch. a-c) Graphs showing relative mRNA levels of IL6, TNF α and MMP-9 of single pinch normalised to mRNA levels in normal skin. mRNA levels of IL6, TNF α and MMP-9 are not upregulated significantly (N=4, $p>0.5$, ns) d-f) Graphs showing mRNA levels of IL6, TNF α and MMP-9 expression of double pinch normalised to mRNA levels in normal skin. All of them were significantly upregulated when compared to normal skin. GAPDH mRNA levels was used as a reference gene (N=4, $p<0.01$, **, Wilcoxon signed rank test, mean (SD))

3.2.15 Pluronic® gel delivery

In preparation for localised delivery of treatment to the insulted region, subcutaneous injection of 30% pluronic gel under the dorsal skin was chosen. To locate and characterise the behaviour of 30% w/v Pluronic® gel macroscopically, Evans blue was used to provide a clear demarcation of the gel, which was observed to be contained in a pocket held towards the skin (Fig. 3.17a-b).

Figure 3.17: Subcutaneous injection of 30% w/v Pluronic® gel. a) Macroscopic image of 100 μ L Pluronic® injected subcutaneously. White demarcation shows how Pluronic® looks from the outside when injected subcutaneously. b) Macroscopic image showing Pluronic® with 3% Evans blue dye being contained under the skin by connective tissue. White demarcation shows how Pluronic® will look if injected subcutaneously.

3.2.16 Cx43asODN was able to penetrate into the different skin layers after subcutaneous administration 1.5 h(I) / 24 h(R).

After observing that Pluronic® gel was contained against the skin, AF488-tagged Cx43asODN in Pluronic® gel was used to investigate the distribution of antisense. AF488-tagged Cx43asODN was not observed in the muscle layer (Fig. 3.18b) but was observed in the skin (Fig. 3.18a, c, d). Small amounts of AF488 tagged Cx43asODN was observed in the upper dermal area after 1.5(I) / 24 h(R) (Fig. 3.18d).

Figure 3.18: AF488 tagged Cx43asODN was able to penetrate into the skin layers 1.5 h(I) / 24 h(R). a) Confocal montage showing AF-488 tagged Cx43asODN observed in the PC region to the upper dermal regions but almost none in the underlying muscle. c-d) Zoomed in pictures of white squares demarcated in c) for easier viewing. Scale bar in a and b = 75 μ m, c and d = 25 μ m.

3.3 Discussion

3.3.1 Summary

It is hypothesised that Cx43 is upregulated during the early events of PU development and is a key driver in PU progression and severity. To be able to investigate this, an experimental model of early PU is required. A review of previous work in this field shows that there is no consensus on the model parameters (Table 3.1). Different investigators use varying durations of I/R, the number of I/R cycles, magnet types (i.e., ceramic, gold-plated), magnet strengths (1,000 gauss, 2,300 gauss), model types

(magnet implantation model, magnet pinch model) to generate an injury suitable for their various experimental applications.

A model for studying early PU should be able to fulfil 3 main criteria. Firstly, the induced injury should be reproducible and also mimic a Stage 1 or earlier PU, in which the epidermis remains intact. Secondly, the induced injury should not be too severe from the onset (i.e., showing features of Stage 2 PU). Thirdly, the injury caused by a single pinch should have the capacity to progress into a more severe phenotype, such as a Stage 2 PU or higher, when a second pinch is applied. Taking these criteria into consideration, the development of the model was approached by first comparing two popular magnet models of experimental PU and later optimising the duration of ischaemia on the chosen model, which would determine the severity of the PU (Fig. 3.1 - 3.4).

Although there have been numerous publications, there have been few detailed histological descriptions of the specific events that occur during early I/R injury where the epidermis is still intact (summarized in Table 3.1). Here, after determining the optimal experimental model conditions, the model was characterised histologically (Fig 3.5 - 3.8). Vascular hyper-permeability and epidermal Cx43 upregulation were also investigated (Fig 3.9 – 3.11). A more severe double pinch I/R model was built upon this model through an additional cycle of I/R, with the aim of using it to test treatment efficacy (Fig. 3.12 – 3.16). The behaviour of Evans blue in Pluronic® and AF488-tagged Cx43asODN in Pluronic® was studied when subcutaneously injected under the insult area (Fig. 3.17).

Below, I will discuss: 1) the model selection, 2) histological observations, 3) vascular leak, necroinflammatory debris, 4) Cx43 upregulation in the epidermis, 5) double pinch I/R model and 6) Cx43asODN subcutaneous injection.

3.3.2 Model selection

3.3.2.1 Surgical implications of the magnet implantation model

The magnet pinch model has multiple advantages over the magnet implantation model, as it is non-invasive, cheaper (two insults generated in 1 mouse), requires less technical expertise, faster, and has fewer complications (e.g., foreign body reaction). In the magnet implantation model, a magnet (or magnetic material) needs to be inserted beneath the skin via an incision. Pro-inflammatory cytokines induced by the incision are potential confounders to the model, complicating the monitoring of inflammation [236]. The implantation necessitates the use of surgical tools and sutures, which requires training. Furthermore, different surgeons may have different surgical skill levels. These factors may result in different outcomes of implantation and consequently, introduce variations (Fig. 3.2a-f).

3.3.2.2 Fibrous reaction observed in magnet implantation model

Surprisingly, the initial developers of the magnet implantation model did not account for any fibrous reaction or response to a foreign body [208]. A fibrous reaction in the magnet implantation model was observed after 1.5 h(I) / 24 h(R) despite the use of sterile gold-plated magnets (Fig. 3.2e). However, publications using the magnet implantation model included a control with the implanted plate which showed normal

skin histology with no foreign body response towards the implanted plate or magnet [213, 215]. To explain this, a recent review by Mariani et al. details the process of foreign body response [237]. Seemingly, a foreign body response is kick-started by a combination of foreign material and blood from damaged blood vessels [237]. Additional inflammation caused by the secretion of pro-inflammatory cytokines (IL-6 and TNF α) by activated macrophages from foreign body response may aggravate the injury [237]. This could also explain why magnet implantation models often show a phenotype where it seems like the damage is bottom-up rather than top-down. This explanation is further supported by comparing a magnet implantation model (Fig. 3.19) and magnet pinch model (Fig. 3.20) both done in rats in other publications with similar durations of ischaemia (4 h(I), 100 mm Hg and 3 h(I), 1000 gauss magnet).

Figure 3.19: Histological images taken from Hashimoto et al., 2008 (Fig. 9). a-d) Histological images showing changes after 4 h(I) / 12 h, 1 d, 3 d, 1 w (R) of magnet implantation model in rats. c) Histological images showing massive leukocyte infiltration in the muscle. Epidermis is still intact. Scale bar = 1 mm

The epidermis was intact throughout the magnet implantation model of Hashimoto et al., (Fig. 3.19) while necroinflammatory debris and epidermal loss were observed by Swisher et al in a pinch model (Fig. 3.20). This comparison suggests that damage starts at the lower layers for the magnet implantation model, but the damage is rampant in the epidermal and dermal skin layers of the magnet pinch model (Fig. 3.20). Although not reported in the publication by Swisher et al., PC damage was also observed in our magnet pinch model (Fig. 3.5t, u & Fig. 3.6t, u.), suggesting that it creates an I/R injury that affects the whole skin. Furthermore, while the magnet pinch model was used on mice in my research, histological similarities such as NID is also seen in the rat magnet pinch model (Fig 3.6d, Fig 3.20f, g). Taking into consideration the advantages of the

magnet pinch model and the disadvantages of the magnet implantation model, the magnet pinch model was determined to be the more suitable model.

Figure 3.20: Histological pictures taken from Swisher et al., 2015 (Fig. 6) showing changes after 1 h(I) or 3 h(I) of magnet pinch model in rats. a-d) Histological images showing skin that underwent 1 h(I) / 1, 2, 3, 9 d(R). e-h) Histological images showing skin that underwent 3 h(I) / 1, 2, 3, 9 d(R). Scale bar = 100 μ m

3.3.3 Vascular abnormalities and necroinflammatory debris

3.3.3.1 Vascular abnormalities

Vascular abnormalities were observed in the skin caused by I/R injury (Fig. 3.9). There was vascular hyper-permeability at the edge of insult (Fig. 3.9, orange box), and a lack of functional blood vessels in the centre of insult (Fig. 3.9, red box). An investigation of the effects of I/R on the heart reported that I/R insulted endothelial cells lose barrier function and result in vascular hyper-permeability [238]. In spinal cord injuries, I/R also occurs as a secondary injury [239]. In a partial laminectomy rat spinal cord injury model, it was observed that I/R, induced vascular hyperpermeability [181]. This

previous work investigating I/R is in line with the observation of vascular hyper-permeability in our single pinch magnet model.

At the centre of insult, there was a lack of perfusion in blood vessels as indicated by the lack of FITC-BSA (Fig. 3.9, red box). This could either be explained by the no-reflow phenomenon or vessel death. In a publication by Nanobashvili et al., “no-reflow” was observed at 2.5 h(I) / 2 h(R) in the hind limb of rabbits [240]. After 30 min of reperfusion, blood flow reached normal levels, probably due to reactive hyperaemia, but kept reducing on a downward trend from 30 min to 270 min of reperfusion [240]. Another publication by Sezer et al. compiled a table on temporal and spatial changes during coronary occlusion [241]. Similarly, after reperfusion, there was an “over-flow” that was followed by normal, slow or “no-reflow in the late phase of reperfusion [241]. Sezer et al. also observed changes taking place in capillaries. During the initial phase of reperfusion, capillaries are obstructed by “plugs” consisting of blood cell aggregates. In the late phase of reperfusion, capillaries remained plugged and were hyper-permeable [241].

3.3.3.2 Necroinflammatory debris

One of the advantages of the single pinch model is that the epidermis is left intact. This allowed the observation of necroinflammatory debris (NID) that may have otherwise been missed. A few areas of the epidermis within the insult exhibited NID. The NID was identified as masses of neutrophils that gathered at the epidermis, identified by their Ly6g neutrophil specific staining (Fig 3.10b). The exact reason for neutrophil accumulation under the epidermis is unknown although it is known that neutrophils are

attracted by damaged cells releasing DAMPs [233]. This observation was also reported in a rat I/R model [227]. In that study, no NID was observed when the skin was put through 1 h of ischaemia. NID was only observed in the epidermis 2 days after 3 h of ischaemia [227]. In the same report, the observation of NID came 2 days later with a longer ischaemia duration when compared to our study. This could be explained by the authors using a weaker magnet (1,000 gauss vs 2,000 gauss). In a human Stage 1 PU, necrosis of the epidermis was described but no observation was made about neutrophils accumulating under the epidermis [28].

3.3.4 Epidermal Cx43 upregulation

3.3.4.1 Cx43, ischaemia reperfusion injury and Chronic wounds

Cx43 is greatly increased in expression in the epidermis and dermis of human venous leg ulcers, diabetic foot ulcers and pressure ulcers [158, 159, 230]. However, the cause of epidermal Cx43 upregulation is still uncertain. Cx43 upregulation in dermal fibroblasts of diabetic foot ulcers is attributed to hyperglycaemia [242]. A review, written by Mustoe, on chronic wounds, suggests that all three major chronic wounds have underlying I/R processes [16]. For example, in venous leg ulcers, the ischaemia event occurs when there is venous insufficiency where normal arterial-venous pressure gradients are not present. This causes blood stasis that eventually leads to ischaemia. Upon elevation of the leg, circulation to the ischaemic area would represent the reperfusion event. This could mean that Cx43 upregulation in all three ulcers may be due to the I/R process at least in part.

Cx43 is up-regulated following stroke, retina ischaemia-reperfusion and spinal cord injury [174, 178, 179]. Down-regulation of Cx43 by Cx43 antisense oligodeoxynucleotide (Cx43asODN) was able to reduce inflammatory responses in organotypic cultures of optic nerves which underwent 2 h of ischaemia and 2 h to 6 d of reperfusion [177]. Selectively blocking Cx43 gap junction communication in myocardial cells and spinal cord astrocytes that have undergone I/R insult reduces the extent of ischaemia-reperfusion injury [172, 173, 179].

In the examples discussed above, Cx43 upregulation is one of the shared observations in chronic wounds and other ischaemia reperfusion models. Connexin biology has not been studied in the context of I/R in the skin. In this study, Cx43 was observed to be up-regulated in the epidermis in the early stages of I/R injury. The observation that Cx43 increases with venous disease progression and ischaemia-reperfusion injury in other situations listed above suggest that Cx43 may have a role to play in disease progression. If Cx43 also has similar roles in experimental PU, downregulating Cx43 could result in the reduction of I/R injury resulting in the prevention of experimental PU.

3.3.5 A more severe experimental PU model: The double pinch model

As an extension to the early PU model, a double cycle model was optimised to produce more severe injuries consistent with a Stage 2 PU. This model can be used to investigate if any treatment works to stop an early experimental PU from progressing into a more severe stage 2 PU.

3.3.5.1 Optimising the double pinch

After characterising the single pinch model with an optimised duration of 1.5 h(I), the double pinch model was developed by testing two limits of reperfusion period (1.5 h(I) / 1.5 h(R) / 1.5 h(I) and 1.5 h(I) / 24 h(R) / 1.5 h(I)). Consistent with the literature, longer periods of reperfusion result in more severe wounds [208]. Nonetheless, results from both durations tested were desirable as they showed a progression. This is important as it would allow us to study the progression and any potential intervention by a treatment.

3.3.5.2 Relating the single pinch and the double pinch

To validate that the double pinch is more severe than the single pinch model, mRNA expression levels of cytokines IL-6, TNF α , and MMP-9 were determined in both models (Fig. 3.15) and the double cycle pinch model was shown to have higher mRNA expression levels of IL-6, TNF α and MMP-9. IL-6 and TNF α findings were consistent with a publication by Kurose et al. that did a genome-wide screening of rat mRNA expression of cytokines during experimental PU. Kurose et al. reported that GM-CSF, IL-1 β , IL1R α , IL-6, IL-10, and TNF α were increased at 12 h [225]. Human Stage 3 and 4 chronic PUs have also increased mRNA expression levels of IL-1 β and TNF α [243]. However, there are conflicting reports on the relationship of IL6, TNF α and MMP-9 regulating Cx43. Epidermal Cx43 was found to be upregulated in our study at 4 h and 24 h but studies done on smooth muscle cells and rat spinal astrocytes, both show that TNF α downregulate Cx43 via a c-jun N-terminal kinase (JNK)-dependent pathway [244, 245]. In agreement with our study, George et al. report that TNF α with increased extracellular calcium significantly increased Cx43 expression in myocytes [246].

Another study also reported Cx43 upregulation in human umbilical vein endothelial cells after 24 h of incubation with TNF α [247]. Heinrich et al. tested various cytokines including IL-6 and TNF α on human bladder smooth muscle cells and found that TNF α or IL-6 led to a significant increase in Cx43 plaques [248]. Cx43 localised to the membrane was also found to be higher after subjecting human bladder smooth muscle cells to TNF α or IL-6 for 48 h [248]. The above publications seem to suggest that the relationship between TNF α , IL6 and Cx43 is not straightforward.

Although there was no publication indicating mRNA expression levels of matrix metalloproteinase (MMP)-2 and MMP-9 in mouse or rat experimental models of PU, one publication reported protein levels of MMP-2 and MMP-9 was increased in experimental PUs in rats on day 7, 14, and 21 [249]. Increased MMP-9 activity has also been correlated with the increasing severity of human chronic wounds [250]. By comparing the magnet single and double pinch, data seems to suggest that mRNA levels of IL-6, TNF α and MMP-9 were elevated in correlation with experimental PU severity.

3.3.6 Cx43asODN in Pluronic® subcutaneous injection

3.3.6.1 Subcutaneous injection of Pluronic® F-127 as a delivery vehicle

Subcutaneous injection was selected to simulate localised delivery to the insulted area. To my knowledge, no publication detailed how and where the treatment was administered and whether it will be present in the localised area until recently in 2018 by Merola et al. In a novel study by Merola et al., hyaluronic acid-based fillers were injected under the skin para-spinally along the dorsum on each side of the vertebral

column [251]. In agreement with our observation (Fig. 3.17), the hyaluronic acid-based fillers remained in the pocket of the injected region [251].

Pluronic® F-127 (PF127) has been widely used to deliver therapeutic proteins, peptides, plasmid DNA and genes [252]. PF127 has been used mainly for sustained release in therapeutic research. In a study on insulin delivery, PF127 was able to prevent the initial burst release and maintained continued release of insulin over 48 h [253]. In another study, a significant increase in the erythropoietin mRNA expression of up to 10 fold was achieved by delivering the erythropoietin gene in PF127 combined with Pluronic® L61 (PL61) to skeletal muscle [254]. In another publication, PF127 was used to deliver lentiviral vector to 293T cells and primary mixed glial cultures from rat and it was determined that PF127 did not affect vector transduction efficiency [255]. This informs us that PF127 is suitable to deliver lentiviral DNA without damaging the vector. The authors then exploited the thermosensitive properties of the gel to anchor the treatment close to its injection site [255]. These reports suggest that PF127 is a suitable vehicle for Cx43 antisense oligo (Cx43asODN). PF127 is also FDA approved and currently used for delivering Cx43asODN in clinical trials.

3.3.6.2 Cx43asODN degradation in tissue

It is known that unmodified oligodeoxynucleotides degrade in the presence of 3' exonucleases in serum and subcellular extracts [256, 257]. However, oligodeoxynucleotides were found not to be degraded in 5% fetal calf serum at 37 °C after 2 h [256]. Although, in undiluted bovine calf serum, oligodeoxynucleotides were degraded in 15 min, indicating that there needs to be a critical amount of 3'

exonucleases to work [256]. Furthermore, in a rat spinal cord injury study by Cronin et al., Förster resonance energy transfer (FRET) compatible CY3-Cx43asODN-CY5 was administered in Pluronic® [258]. The Cx43asODN was able to diffuse distances of 300-400µm in 2 h and were taken up into cells by 3 h [258]. The Cx43asODN was also able to elicit at >90% knockdown of Cx43, showing that the Cx43asODN was still intact and functional [258].

3.3.7 Conclusion

The magnet pinch model was selected mainly based on its reproducible results after 1.5 h(I). It does not have complications arising from an incision or a foreign material below the insult. Furthermore, two insults can be generated on the mice instead of one. Next, the maximum duration of ischaemia was tested to generate an insult not resulting in a wound. Testing ischaemia duration of 1.5 h, 2 h, 2.5 h and 3 h followed by 24 h of reperfusion showed that 1.5 h(I) did not result in a wound, but the next duration tested resulted in varying levels of injury. This result suggests that 1.5 h(I) was suitable for generating an insult similar to a Stage 1 or earlier PU. This was further confirmed by histological analysis showing different cell populations within the tissue sustained damage, which resulted in epidermal spongiosis, dermal cell loss, PC damage, ECM loss and leukocyte infiltration at 1.5 h(I) / 24 h(R). Moreover, leukocytes and vascular hyperpermeability were found in the insulted tissue. Despite the damage after the insult, the epidermis stayed intact, indicating that the magnet pinch model using a 1.5 h(I) duration was useful to study what happens to the skin in the early processes of PU formation. With the epidermis intact, Cx43 was found to be increased in the epidermis

at 1.5 h(I) 4 h(R) and 24 h(R). The increase of Cx43 levels in the epidermis provided a treatment target for downregulation.

Although the single I/R cycle gave desirable outcomes, a more severe injury was needed to test treatment efficacy. A more severe injury was created by introducing another ischaemia cycle with different reperfusion durations. Reperfusion timings of 1.5 h(R), 4h(R) and 24 h(R) both resulted in more severe injuries and a breakdown of the epidermis. This is further supported by increased mRNA levels of pro-inflammatory cytokines TNF α and IL6 when compared to a single I/R cycle. The mRNA levels of MMP-9 involved in I/R injury were also increased compared to a single I/R cycle.

With the experimental PU model characterised, the delivery of Cx43asODN in Pluronic® gel under the insult area was investigated. Subcutaneous delivery of Cx43asODN in Pluronic® gel was demonstrated to be able to remain under the insult by observing Pluronic® coloured with Evans blue. Cx43asODN was also investigated for its ability to diffuse into the insulted tissue by observing AF488-tagged Cx43asODN to ensure the location.

In conclusion, this chapter lays the groundwork needed to establish I/R parameters to allow the testing of treatments. With this in place, Cx43 downregulation by Cx43asODN and its effects can be investigated in the next Chapter.

Chapter 4. Cx43asODN Treatment of ischaemia reperfusion injury

4.1 Introduction

Currently, significant effort to prevent the occurrence of PUs is centred on recognising PU early, padding or repositioning for pressure relief, managing nutrition and skin microclimate [259]. However, PUs still develop despite following prevention guidelines [259]. Numerous pharmaceutical approaches aimed at preventing pressure ulcer progression have been tried, however, their effectiveness remains inconclusive [196, 260]. There are pharmaceutical strategies to heal a PU, but there is currently no effective therapeutic intervention to prevent PU progression [31].

4.1.1 DNA antisense oligo technology

4.1.1.1 Mechanism of antisense oligo technology

The use of antisense oligonucleotide (ASO) dates back 40 years where Zamecnik inhibited Rous sarcoma virus replication by using 13 nucleotides complementary to the 3' and 5' terminal sequences of Rous sarcoma 35S RNA [261]. The interaction of the 13 nucleotides with 35S RNA interfered with processes involved in viral production and cell transformation [261]. Forty years later, the use of nucleotides to regulate genes to treat diseases is now possible. A review by Shen and David listed ASO's that have undergone clinical trials, which included several FDA approved antisense drugs [262].

The charged nature of DNA and RNA make oligos unlikely to enter the cell through the cell membrane. However, uptake of single-stranded oligos is possible through a

process called gymnosia [263, 264]. Advantages of gymnosia include avoiding toxic effects of transfection agents and compatibility to transfection resistant cell lines. The main disadvantage of this method is the relatively high concentrations of ASOs required (~ 0.1 - $10 \mu\text{M}$ vs. 1 - 10 nM when a transfection agent is used) [262].

Antisense oligonucleotides can be designed to interfere with translation. Translation can be blocked by either ASO-RNase H or ASO-AGO2 mediated mRNA cleavage, depending on the type of ASO (DNA ASO for RNase H and dsRNA for AGO-2) [262]. Only the mechanism of mRNA silencing by RNase H will be discussed here as DNA antisense oligos will be used in the study. The RNase H enzyme recognises and cleaves the mRNA in the DNA: RNA hybrid. Cytoplasmic RNase H can also degrade mature mRNA, resulting in the inhibition of translation [265, 266].

Antisense therapies can utilise either an unmodified DNA antisense oligomer (natural phosphodiester backbone) or a chemically modified one. One characteristic of unmodified antisense is that it degrades very quickly in the presence of 3' exonucleases in serum [256, 257] and hence intravenous administration is not possible. Chemical modifications have been made to delay degradation, mainly by altering the phosphodiester backbones from $\text{P}=\text{O}$ to $\text{P}=\text{S}$ [267]. Although these modified DNA oligomers have a much longer half-life, they may have side effects such as activating the complement pathway [268]. A variety of side effects have been reported in human clinical studies, such as transient thrombocytopenia, fatigue, fever and complement activation [269]. These factors should be considered before deciding the need for antisense oligo modification.

4.1.2 Regulating connexins in I/R

Several studies have been published, reporting the effects of blocking connexin communication on the brain, heart, and spinal cord in an attempt to reduce injury [235]. In the brain, non-specific gap junction blockers, such as octanol, carbenoxolone and 18 α -glycyrrhetic acid, have been used to block gap junction communication. In a rat model of mid-cerebral artery occlusion (MCAO)/ reperfusion, intraperitoneal injection of octanol 30 min before MCAO reduced the infarct size of rat brains by 50% [270]. Octanol was also used to treat another I/R model in rats in which the common carotid arteries were clamped. Here they observed a 30-50% reduction in cell death in the hippocampus [164]. Other non-specific gap junction blockers, carbenoxolone and 18 α -glycyrrhetic acid were also used to reduce cell death by 27% in a transient (18 min) bilateral global ischaemia model in rats [271]. Treatments were effective when non-specific gap junction blockers were administered 30-40 min after the ischaemic event but were not effective if administered 90 min after [271]. In another MCAO model on ICR-1 mice, Gap19 reduced brain infarct volume and Cx43 expression 24 h after cerebral I/R injury [272]. Gap19 was also able to restore astrocyte cell viability after oxygen-glucose deprivation (OGD) [272]. These studies suggest gap junctional communication is involved in the spread of cell death following I/R.

In heart I/R models, studies also centred on blocking gap junction communication, one study subjected extracted mouse cardiomyocytes to 1 h of OGD and 15 min of reperfusion and showed that mimetic peptide Gap19 prevented cardiomyocyte swelling and promoted survival [273]. Gap 19 also reduced infarct size in mouse hearts in vivo after 30 min of ischaemia and 120 min of reperfusion [173]. In an I/R model of the heart in rats following 40 min of ischaemia and two days of reperfusion, mimetic

peptides Gap26 and Gap27 reduced myocardial infarct size [274, 275]. An interesting study reduced myocardial infarct size by replacing Cx43 with Cx32 [276]. Partial depletion of myocardial Cx43 also reduced infarct size in one report [277] but not in two other studies [278, 279]. This suggests that reduction of Cx43 expression may not always result in I/R injury reduction in the heart.

There are also studies on down-regulating Cx43 following spinal cord injury. As mentioned in Chapter 1 (section 1.12), Cx43 is implicated in spinal cord injury. By using Cx43asODN on a rat spinal cord compression model, rats have better recovery of locomotion from 24 h to 4 weeks. Cx43asODN treatment resulted in decreased spinal cord swelling, disruption, astrocyte activation, and neutrophil recruitment [181]. This was confirmed using mice with two transgenic mutations. These mice have astrocytes that were unable to express Cx43 (astrocyte Cx43^{-/-}) and Cx30 (Cx30^{-/-}). Astrocyte Cx43^{-/-}, Cx30^{-/-} mice had improved locomotor scores from 3 days post-injury and continued to 56 days [280]. The transient prevention of the opening and/or docking of hemichannels with Peptide5, for 24 h also improved locomotor recovery in a spinal cord weight drop injury model. Treatment with Peptide5 decreased astrocyte activation in surrounding tissues adjacent to the lesion site and the number of active microglia [281]. TNF α and IL1 β were also reduced by Peptide5 treatment, suggesting that blocking Cx43 hemichannels has an anti-inflammatory effect [281].

In summary, various publications have shown that Cx43 is involved in I/R injury. Cx43 was shown to be an important target to reduce I/R injury when Cx43 gap junction blockers, Cx43^{-/-} or Cx43asODN were used. This work suggests that inhibiting Cx43

gap junction communication or down-regulating Cx43 may reduce the extent of I/R injury in the skin.

4.1.3 HMGB1 and ischaemia reperfusion injury

Damaged and necrotic cells resulting from I/R can release HMGB1 [282]. HMGB1 is a well-known damage-associated molecular pattern (DAMP) that has shown to be involved in I/R injury in many studies such as kidney [283], liver [284], brain [285], heart [286] and the eye [287]. HMGB1 can also be increased in serum after liver I/R and cause injury to the lung [273]. In all mentioned organs, HMGB1 was up-regulated and if inhibited, reduces I/R injury to the target organ [273, 283-287]. Although HMGB1 is also involved in endotoxemia and microbial infection [288, 289], HMGB1 is released more rapidly in I/R injury. In myocardial I/R injury, HMGB1 is significantly higher just 30 min after ischaemia and peaks at 6 h post ischaemia [290] compared to releasing at 8 to 10 h after TNF stimulation in pituicytes [291] and 8 h in macrophage cultures [289] or 16 h after LPS administration in mice [289]. Necrotic or damaged cells passively releasing HMGB1 upon I/R injury can explain why HMGB1 is an early mediator of I/R injury.

4.1.4 Necroptosis and ischaemia reperfusion injury

Necroptosis, a relatively new cell death pathway compared to apoptosis and necrosis, is involved in I/R injuries in many organs such as the brain [292, 293], heart [294], and kidney [295]. In contrast to necrosis, necroptosis is regulated by receptor-interacting protein kinase 3 (RIP3/RIPK3) [296]. When this pathway is activated, RIP3 is liberated from the RIP1/RIP3 complex and recruits MLKL to the cell membrane to create pores leaking cell constituents into the extracellular space causing the cells to die [297]. In

comparison to apoptosis, necroptosis is an inflammatory process that includes the release of HMGB1 that results in inflammation. [298]. It has been suggested that RIP3 is an indispensable protein for necroptosis [299]. This is supported when murine cytomegalovirus infection activates a RIP3-dependant necroptosis, which is independent of RIP1 activation.

4.1.5 Summary

The reduction of Cx43 gap junction expression by antisense has been reviewed above. In topical applications, the instability of unmodified antisense molecules in serum is not an issue but rather an advantage by limiting off-target systemic exposure. This has led to the development of unmodified antisense targeting Cx43 such as Nexagon which is in Phase II clinical studies [300]. Cx43 is implicated in spreading damage in various I/R models and human late-stage PU. Furthermore, epidermal Cx43 was observed to be upregulated at 1.5 h(I) / 4 h(R), 24 h(R) in the previous chapter. Both HMGB1 and RIP3 will be investigated as it has been reported to occur in I/R injury models.

4.1.6 Hypothesis

I hypothesise that by reducing the I/R-induced increase of epidermal Cx43 expression using Cx43asODN, I/R injury will be reduced by suppressing necroptosis.

4.1.7 Aim

This chapter aims to show that Cx43asODN can prevent the increase of epidermal Cx43 after I/R and to show that I/R injury and necroptosis can be reduced by Cx43asODN

treatment in a single pinch I/R mouse model followed by validation in a double pinch model.

4.2 Results

4.2.1 Cx43asODN treatment prevents the increase of epidermal Cx43 protein levels after 1.5 h(I) / 4 h(R)

After 1.5 h(I), Cx43asODN in Pluronic® gel or vehicle control was injected subcutaneously under the region of insult. After 1.5 h(I) / 4 h(R), the skin was harvested, and epidermal Cx43 protein expression was measured. Epidermal Cx43 was found to have increased by 4.24 times in the insulted skin with vehicle control (VC) compared to normal skin (Fig. 4.1a-c). The increase of epidermal Cx43 was significantly reduced (Fig. 4.1g) in the Cx43asODN treated group (TR), which were similar to normal levels (Fig. 4.1d). This suggests that Cx43asODN was able to prevent I/R-induced up-regulation of epidermal Cx43.

Figure 4.1: The increase of Cx43 after 1.5 h(I) / 4h(R) is prevented. a, e) Confocal images showing Cx43 (red) protein levels in the normal epidermis, b) vehicle control (d) Cx43asODN treated insulted epidermis. c) Graph showing quantified Cx43 signal of the normal epidermis and VC epidermis. Cx43 was significantly increased after 1.5 h(I) / 4 h(R) (N=5, $p < 0.01$, Mann-Whiney U-test). d) Graph showing quantified Cx43 signal of the normal epidermis and Cx43asODN treated insulted epidermis. Epidermal Cx43 was not increased at 1.5 h(I) / 4 h(R) (N=6, $p < 0.01$, Mann-Whiney U-test). g) Graph showing quantified Cx43 signal of VC epidermis and Cx43asODN treated insulted epidermis normalised to its respective normal epidermis and compared to each other. Increase in epidermal Cx43 was significantly reduced when treated with Cx43asODN 1.5 h(I) / 24 h(R) (N=5 (VC skin), N=6 (Cx43asODN treated insulted skin), $p < 0.01$, Mann-Whitney U-test). Scale bar = 10 μ m

4.2.2 Cx43asODN treatment prevented epidermal condensed nuclei after 1.5 h(I) / 4 h(R)

After observing the prevention of I/R-induced Cx43 up-regulation after 1.5 h(I) / 4 h(R), I investigated whether or not histological changes could be prevented. In VC skin, epidermal nuclei abnormal condensation (Fig. 4.2a) were similar to that characterised after 1.5 h(I) / 4 h(R) in the previous chapter (Fig. 3.5e). These abnormal nuclei condensation in the epidermis was not observed in the Cx43asODN treated skin, suggesting that the injury caused by the insult was prevented (Fig. 4.2b). However, leukocyte infiltration was observed in VC and treated dermis (Fig 4.2c-f). The PC was also observed to be damaged in both conditions (Fig 4.2e, f). No obvious benefit from Cx43asODN treatment was observed in the dermal and hypodermal regions at 4 hours after insult (Fig. 4.2c-f).

Figure 4.2: Cx43asODN treated insulted skin after 1.5 h(I) / 4 h(R) prevented epidermal nuclei abnormal condensation. Representative widefield pictures of H&E staining of vehicle control and Cx43asODN administered histology of epidermis, dermis, and hypodermis in insulted skin. a) Epidermis shows abnormal nuclei condensation which is b) prevented by Cx43asODN treatment. c-f) Dermis and hypodermis showed no obvious differences in leukocyte infiltration and damage to the PC resulting from Cx43asODN treatment. N=5 (VC skin), N=6 (Cx43asODN treated insulted skin). Scale bar = 50 μ m.

4.2.3 Cx43asODN prevented epidermal spongiosis, loss of dermal cells and PC degradation after 1.5 h(I) / 24 h(R)

After 1.5 h(I) / 24 h(R), in VC treated insults, spongiosis (Fig. 4.3a, 3.5e), loss of dermal cells (Fig. 4.3c, 3.5q) and muscle degeneration (Fig. 4.3e, 3.5u) were observed. In contrast, following the Cx43asODN treatment epidermal spongiosis was not observed (Fig. 4.3b). Moreover, more dermal fibroblast was seen, and their morphology was better than vehicle control (Fig. 4.3c-d) and the PC muscle fibres were less damaged, as indicated by the presence of muscle nuclei (Fig 4.3d, f).

Figure 4.3: Cx43asODN treated insulted skin after 1.5 h(I) / 24 h(R) prevented epidermal spongiosis, loss of fibroblast and loss of muscle nuclei. Representative widefield pictures of H&E staining of vehicle control and Cx43asODN treated insulted skin histology of epidermis, dermis, and hypodermis. a) Vehicle control epidermis shows spongiosis, c) the lack of dermal cells and e) loss of muscle nuclei. b) Cx43asODN treated epidermis shows no spongiosis, d) presence of dermal fibroblast and f) muscle fibres with nucleus still present. N=5 (VC skin), N=6 (Cx43asODN treated insulted skin). Scale bar = 50 μ m.

4.2.4 Cx43asODN treatment reduces neutrophil infiltration to the dermis after 1.5 h(I) / 4 h(R)

In I/R injury, sterile inflammation plays a role in removing dead cells but may also damage cells. Since neutrophil infiltration was observed in the dermis of this I/R skin model, it was important to determine if Cx43asODN treatment was able to reduce neutrophil infiltration in the dermis. Neutrophils stained with a neutrophil specific Ly6g antibody (visualised in red) and were observed to infiltrate the VC skin (Fig. 4.4b) and this was reduced in Cx43asODN treated skin (Fig. 4.4c, d).

Figure 4.4: Cx43asODN treated skin after 4 h post 1.5 h of ischaemia had fewer neutrophil infiltrates. Representative confocal images of neutrophils stained in red with Ly6g antibody. White arrows point to neutrophils. a) Normal skin shows almost no neutrophils and in contrast, b) Neutrophils were observed to have infiltrated in VC skin. c) Cx43asODN treated insulted skin shows fewer neutrophils infiltrating the insulted region. d) Graph of total neutrophils counted per 1000px² showing VC skin having a significant increase (N=5, $p < 0.01$, Kruskal-Wallis, Dunn's post hoc test). Cx43asODN treated skin have a non-significant increase of neutrophils compared to normal skin. (N=5, $p > 0.5$, Kruskal-Wallis, Dunn's post hoc test). Scale bar = 25 μ m

4.2.5 HMGB1 protein levels are significantly increased after 1.5 h(I) / 4 h(R)

Epidermal nuclei condensation, neutrophil infiltration and accumulation at the epidermis were observed after 1.5 h(I) / 4 h(R) (Fig. 3.5, 3.10) so I looked for changes in epidermal HMGB1 protein levels as it is an early mediator of cell death and cytokine expression in I/R injury. Skin was stained with HMGB1 (in red) after 1.5 h(I) / 4 h(R). HMGB1 protein signal was found to be significantly increased in the epidermis of VC skin compared to normal skin (Fig. 4.5a-b, e). However, after 1.5 h(I) / 24 h(R), the epidermal HMGB1 protein signal was no longer significantly increased when compared to normal skin (Fig. 4.5c-d, f).

Figure 4.5: Epidermal HMGB1 is significantly increased after 1.5 h(I) / 4 h(R). Panel of representative confocal pictures showing HMGB1 stained in red of a) normal skin after 1.5 h(I) / 4 h(R), b) VC skin after 1.5 h(I) / 4 h(R), c) normal skin after 1.5 h(I) / 24 h(R) and d) VC skin after 1.5 h(I) / 24 h(R). Graphs showing quantified epidermal HMGB1 protein signal after e) 1.5 h(I) / 4 h(R) showing significant increase (N=6, $p < 0.01$, Wilcoxon signed-rank test) and f) showing no significant increase 24 h later. (N=5, $p > 0.05$, ns, Wilcoxon signed-rank test). Scale bar = 10 μ m.

4.2.6 Cx43asODN prevents the increase of HMGB1 protein levels after 1.5 h(I) / 4 h(R)

HMGB1 protein levels, after vehicle control after 1.5 h of ischaemia. then 4 h(R), epidermal HMGB1 increased (Fig 4.6a-b). In comparison, HMGB1 was significantly reduced with Cx43asODN treatment (Fig 4.6d, e).

Figure 4.6: The increase of epidermal HMGB1 after 1.5 h(I) / 4 h(R) is prevented by Cx43asODN treatment. Representative confocal images showing HMGB1 (red) protein levels in the epidermis of a) normal skin and its b) VC skin and c) normal skin and d) Cx43asODN treated insulted skin after 1.5 h(I) / 4 h(R). e) Graph of HMGB1 signal of VC and Cx43asODN treated insulted skin normalised to levels in the normal skin and compared to each other. Cx43asODN significantly prevented the increase of epidermal HMGB1. (N=6 (VC skin) N=7 (Cx43asODN treated insulted skin), $p < 0.05$, Mann-Whitney U-test). Scale bar = 10 μ m

4.2.7 HMGB1 protein levels remain close to normal levels in Cx43asODN treated insulted skin after 1.5 h(I) / 24 h(R)

Cx43asODN treatment prevented the increase of epidermal HMGB1 protein levels after 4h (R), After 1.5 h(I) / 24 h(R), epidermal HMGB1 declined from an increased level at 4 h post ischaemia in VC skin (Fig 4.7a-b). In Cx43asODN treated skin, HMGB1 protein signals were found to be comparable to those found in normal skin (Fig 4.6c-d, f). When comparing HMGB1 protein signal in vehicle control and Cx43asODN treated skin, there was no significant difference after 24 h(R) (Fig. 4.7e)

Figure 4.7: Epidermal HMGB1 protein level after 1.5 h(I) / 24 h(R) did not increase after Cx43asODN treatment. Confocal images showing HMGB1 (red) protein levels in the epidermis of a) normal skin and its b) VC skin, and c) normal skin and d) Cx43asODN treated skin. e) Graph of epidermal HMGB1 protein levels in VC and Cx43asODN treated skin normalised to HMGB1 protein levels in the normal skin and compared to each other. f) Graph of epidermal HMGB1 protein levels showing Cx43asODN treatment did not result in the up regulation of epidermal HMGB1 protein levels after 1.5 h(I) / 24 h(R). (N=5 (VC skin), N=6 (normal skin and Cx43asODN treated insulted skin), $p>0.05$, Mann-Whitney U-test). Scale bar = 10 μ m

4.2.8 Epidermal RIP3 protein levels increase with I/R progression after 1.5 h(I) / 4 h(R) and 24 h(R)

RIP3 protein in the skin is responsible for necroptosis and is reported to be up-regulated in I/R injuries. Skin tissues were stained with RIP3 antibody. After 1.5 h(I) / 4 h(R) and 24 h(R), RIP3 protein signal was increased in VC epidermis compared to normal epidermis (Fig. 4.8a-b). However, RIP3 was only significantly increased after 24 h of reperfusion (Fig. 4.8c) and not after 1.5 h(I) / 4 h(R) (Fig. 4.8b)

Figure 4.8: Epidermal RIP3 is significantly increased after 24 h post 1.5 h of ischaemia. Panel of confocal pictures showing RIP3 staining in red of a) normal skin, b) VC skin after 4 h (R), c) VC skin at 24 h post ischaemia. d) Graph showing significant increase in epidermal RIP3 protein signal at 24 h post ischaemia when compared to normal skin (n=3 normal epidermis, n=6 (4 h post ischaemia vehicle control skin) n=4 (24 h post ischaemia vehicle control skin), $p < 0.05$, Kruskal-Wallis, Dunn's post hoc test). Scale bar = 50 μ m

4.2.8 Cx43asODN prevents the increase of RIP3 protein levels during I/R progression after 1.5 h(I) / 4 h(R) and 24 h(R)

To investigate if the treatment of Cx43asODN has any effect on RIP3 protein levels, Cx43asODN and its vehicle control were administered after 1.5 h of ischaemia. After 1.5 h(I) / 4 h(R), comparable epidermal RIP3 protein signals, were observed between VC and Cx43asODN treated insulted epidermis (Fig 4.9a-c, g). However, after 1.5 h(I) / 24 h(R), epidermal RIP3 protein signal was significantly reduced in Cx43asODN treatment compared to vehicle control where it was elevated (Fig. 4.9d-f, h).

Figure 4.9: Cx43asODN treatment prevented the increase of epidermal RIP3 after 1.5 h(I) / 24 h(R). Panel of representative confocal images showing epidermal RIP3 staining in red of a) normal skin, b) VC skin, c) Cx43asODN treated skin at 1.5 h(I) / 4 h(R) and d) normal skin, e) VC skin, f) Cx43asODN treated skin after 1.5 h(I) / 24 h(R) g) Graph of quantified epidermal RIP3 protein signal after 1.5 h(I) / 4 h(R) of normal skin, VC skin and Cx43asODN treated skin showing no significant increase. N=8 (normal epidermis), N=6 (1.5 h(I) / 4 h(R) vehicle control skin), N=9 (1.5 h(I) / 4 h(R) Cx43asODN treated insulted skin), $p>0.05$, Kruskal-Wallis, Dunn's post hoc test). h) Graph of quantified epidermal RIP3 protein signal at 1.5 h(I) / 24 h(R) of normal skin, VC skin and Cx43asODN treated skin showing a significant reduction of epidermal RIP3 protein signal with an increase in VC (N=8 normal epidermis, N=4 (1.5 h(I) / 24 h(R) post ischaemia vehicle control skin), N=5 (24 h post ischaemia Cx43asODN treated insulted skin), $p<0.01$, Kruskal-Wallis, Dunn's post hoc test). Scale bar = 50 μm .

4.2.9 Cx43asODN prevents PU progression and reduces I/R injury

Cx43asODN treatment in the single pinch model reduced the increase in epidermal Cx43, epidermal abnormal nuclei condensation, neutrophil infiltration, HMGB1 and RIP3 expression. These observations suggest that Cx43asODN treatment had the potential to prevent the progression of the Stage 1 PU to a more severe PU stage.

This intervention was tested on the double pinch model, which results in a more severe I/R injury where the epidermis is lost. Macroscopic images show that Cx43asODN treated skin produced less severe injuries (Fig 4.10a) compared to its control. The peak of progression was at days 4 and 5 and was significantly less severe in the Cx43asODN group compared to its control (Fig. 4.10b, $n=3$, $p<0.05$). In particular, by day 4, only 60% of Cx43asODN treated skin progressed into an open wound as compared to 100% of Cx43senseODN control skin (Fig. 4.10c, $n=10$, $p<0.05$). In addition, by day 10, 50% of Cx43asODN treated insults are fully healed compared to only 10% of Cx43senseODN treated skin (Fig 4.10d).

Figure 4.10: Cx43asODN treatment reduces I/R injury progression in a double pinch model of 1.5 h(I) / 4 h(R) / 1.5 h(I) / 15 d(R). a) Representative macroscopic picture of insulted skin that underwent a double pinch model over 11 days. Control treated skin seems to only heal by day 11 as opposed to Cx43asODN treated skin which heals by day 6. Red boxes indicate injury phase and green box indicated that it is healed. b) Pictures are scored by 3 independent blinded researchers and Cx43asODN treated skin is significantly less severe on days 4 and 5. (N=3, $p < 0.05$, Two-way ANOVA, Bonferroni test) c) Data was plotted as a survival curve and by day 4, 60% Cx43asODN treated skin have open wounds as compared to the 100% of control administered skin. This is a significant decrease. (N=10 (5 animals, 2 wounds each), $p < 0.05$, Log-rank (Mantel-Cox) test) d) Data was plotted in a Kaplan Meier curve, documenting open wounds, and by day 10, 50% of Cx43asODN treated mice are fully healed as compared to just 10% of the control mice. These curves are also significantly different. (N=10 (5 animals, 2 wounds each), $p < 0.01$, Log-rank (Mantel-Cox) test).

4.3 Discussion

4.3.1 Summary of key findings:

A single dose of 300 μ M Cx43asODN subcutaneously administered under the insult region resulted in the prevention of 1) the increase of epidermal Cx43 protein, 2) preserving dermal and muscle cells, 3) reduction of neutrophil infiltration, 4) prevention of elevation of epidermal HMGB1 and 5) prevention of elevation of epidermal RIP3 in an experimental early PU model of a single pinch of 1.5 h of ischaemia. Further investigation of the Cx43asODN treatment found it to be able to reduce the progression of experimental PU in an I/R double pinch mice model.

The results will be discussed in two main parts: 1) prevention of elevation of Cx43 protein level and histological observations, 2) down-regulation of RIP3 and preventing PU progression.

4.3.2 Prevention of the elevation of Cx43 protein levels and histological observations

This study is the first to observe and measure the effects of Cx43asODN on epidermal Cx43 in the context of an early pressure ulcer. Using the single pinch model of 1.5 h of ischaemia, 4 h after Cx43asODN treatment, epidermal Cx43 up-regulation was found to be reduced (Fig. 4.1). This prevention of epidermal Cx43 elevation also resulted in keratinocytes that looked similar to normal skin Fig. 4.2). The effects of Cx43asODN treatment were also monitored at 24 h and I found that spongiosis was prevented and more dermal fibroblasts were preserved compared to the vehicle control (Fig. 4.3). These findings confirmed that preventing epidermal Cx43 up-regulation was associated with preserving histological normality in the epidermis.

4.3.2.1 Epidermal Cx43 regulation in chronic wounds

No other study has examined epidermal Cx43 in early PUs. Connexin regulation in other contexts, such as human diabetic ulcers will be considered as diabetic ulcers are similar to pressure ulcers in the context of I/R being one of the drivers of injury in the skin.

In the normal wound healing process, wound edge cells downregulate Cx43 in the first 24-48 hours as cells become migratory (Coutinho et al.2003, Qui et al 2003, Mori et al 2007). In a streptozotocin (STZ) diabetic rat wound model, an abnormal increase in epidermal Cx43 and the formation of a non-migratory bulb of keratinocytes was observed at the wound edge (day 1) [151]. By treating these diabetic wounds with Cx43asODN, abnormal epidermal Cx43 expression at the wound edge was prevented [151]. This resulted in faster re-epithelisation in the diabetic rat wound [151]. It has been suggested that Cx43 interacts with P120ctn/Rho GTPase, which could be important for the migration of keratinocytes [301]. The abnormal increase of epidermal Cx43 expression in the diabetic rat wound edge is also seen in human diabetic foot ulcers [158, 302]. Cx43asODN treatment (Nexagon) is in phase II clinical trials for diabetic ulcers [300]. Preventing the abnormal increase of epidermal Cx43 is essential for the wound edge cells to become migratory and close the wound. Kretz et al. reported that the epidermis of Cx43 deficient mice migrated faster and closed 1 day faster than normal controls [303]. Mori et al. also reported that downregulation of Cx43 by Cx43asODN at the wound site led to reduced neutrophil recruitment over 2 days [160]. This was accompanied by a significant reduction of Ccl2 and TNF α [160]. In this study, preventing the up-regulation of epidermal Cx43 with Cx43asODN in early PU has similar beneficial effects.

4.3.3 Regulating necroptosis and preventing PU progression

In the Cx43asODN treatment group, fewer neutrophils infiltrated the insulted region of the skin that underwent 1.5 h(I) / 4 h(R) injury (Fig. 4.4). This reduced number of neutrophils may indicate that fewer cytokines or chemokines were being produced which could be due to Cx43asODN reducing cell death. While the literature has pointed to apoptosis [304], this is counter-intuitive due to the inflammatory nature of the injury, as apoptosis is generally a non-inflammatory process [304]. Necrosis was suspected and led to the interest in HMGB1, which is a marker of necrosis [305]. Epidermal HMGB1 was found to be up-regulated in the skin that has been subjected to 1.5 h(I) / 4 h(R) (Fig. 4.5). This up-regulation of HMGB1 was prevented in epidermal cells after Cx43asODN treatment (Fig. 4.6). The observation that the injury was progressive suggested some form of regulated cell death, one of which is necroptosis [306]. A driver protein of necroptosis, RIP3, was found to be up-regulated in the epidermis after 1.5 h(I) / 4 h(R) and 24 h(R), which suggested that these cells were primed for necroptosis (Fig 4.8). This up-regulation of RIP3 was also prevented by Cx43asODN treatment (seen at 4 h and 24 h (Fig. 4.9)). This up-regulation of epidermal Cx43, HMGB1 and RIP3 were all prevented by Cx43asODN treatment. This suggested that Cx43asODN treatment had the potential to prevent the progression of the Stage 1 PU to a more severe PU stage (i.e., where the epidermis is lost). This was later confirmed when a single dose of Cx43asODN treatment was able to reduce I/R injury and limit the progression of the experimental PU (Fig. 4.10). The following discussion addresses the necrosis and necroptosis findings.

4.3.4 Dermal fibroblast and panniculus carnosus

From Figures 4.2 and 4.3, initially at 4 h, the number of dermal cells seems to be the same for treated and untreated skin which underwent 1.5 h of ischaemia. However, at 24 h, the treated skin contained more dermal cells than untreated skin. This is similar for the satellite cells of the panniculus carnosus where the cells are still present at 4 h regardless of treatment but was preserved only in the Cx43asODN treated skin (Fig. 4.2, 4.3).

Glass et al. reported that Gap27 mimetic peptide prevents cell death signals from spreading after ischaemia reperfusion in fibroblasts by blocking gap junction intercellular communication (GJIC) and reducing Cx43 protein levels [307]. Cell viability was also preserved after ischaemia reperfusion when fibroblast was transduced with Cx43shRNA [307]. Many publications describe increased proliferation and migration of dermal cells when Cx43 is reduced or blocked [159, 160, 308, 309]. Mori et al. found that the number of fibroblasts in the wound bed was significantly increased in Cx43asODN treated mice and suggested that this was due to an influx of fibroblasts [160]. This was supported by an in-vitro scrape-wound assay which showed that there was an enhanced rate of fibroblast migration when Cx43 was knocked down [160]. Cogliati et al. used Cx43^{+/-} mice to show that Cx43 deficiency increases dermal fibroblast proliferation and activation after wounding [308]. Mendoza et al. reported that fibroblasts transfected with Cx43shRNA, to knock down Cx43, showed faster migration speeds and Cx43asODN treated fibroblasts had enlarged lamellipodia [159]. Wright et al. used Gap27, a GJIC blocker, on dermal fibroblast to produce increased migration rates compared to controls [309]. When taken together, our observations of

more dermal fibroblast in Cx43asODN treated skin is expected and agrees with the literature.

4.3.5 Elevated epidermal HMGB1 in early PU

Epidermal HMGB1 was found to be 2.1x higher than normal epidermis after 1.5 h(I) / 4 h(R) and was reduced again by 1.5 h(I) / 24 h(R). HMGB1 is up-regulated in just 30 min after ischaemia in the heart, peaking at 6 h post ischaemia and then decreasing [290]. This is consistent with the observation that HMGB1 is increased at 4 h of reperfusion, but not 24 h reperfusion, after being subjected to 1.5 h of ischaemia (Fig. 4.5).

Cx43asODN treatment prevented the increase of HMGB1 protein by a factor of 2. This could be due to the reduced spreading of death signals as Cx43 up-regulation was prevented by Cx43asODN treatment. However, there is also a possibility that Cx43 directly regulates HMGB1. In one publication on Cx43 interactors related to osteoarthritis, Gago-Fuentes et al. showed that HMGB1 could be one of the Cx43-interacting proteins in moderate osteoarthritis (grade III) [310]. It is important to note that Cx43 – HMGB1 interaction is not detected in healthy or early osteoarthritis (grade I). Besides this publication, reviews on Cx43 interacting proteins did not report Cx43 – HMGB1 interaction and this remains to be discovered [311, 312]. Some modifications to Cx43 such as phosphorylation or dephosphorylation may be required before it can interact with HMGB1 [313]. Interactions could also be prohibited due to locational differences in cellular space (Cx43 located in lipid rafts while HMGB1 is normally in

the nucleus). However, these are all speculations and there is no other publication suggesting that Cx43 directly regulates HMGB1.

4.3.6 Regulating epidermal RIP3 in early PU

Epidermal RIP3 was observed to be 1.82x higher than normal after 1.5 h(I) / 4 h(R) and continued to up-regulate to be 2.29x higher than normal after 1.5 h(I) / 24 h(R). This continued up-regulation of RIP3 has been reported in hippocampal neuronal cultures, which have undergone 2 h of OGD and 7 h or 24 h of reperfusion. RIP3 expression was found to be much higher at 24 h than 7 h post-OGD [314]. Yuan et al. reported that the increased expression of necroptosis proteins RIP1 and MLKL was also prevented in kidney cells when treated with gap junction blocker Gap26. Here, epidermal RIP3 upregulation was not only prevented but was reduced in epidermal cells in the Cx43asODN treated group (Fig. 4.9).

[315]This is surprising as no publications are suggesting a direct regulatory link between RIP3 and Cx43 [129, 311]. However, there could be a possibility that Cx43 plays a direct role in regulating RIP3 as RIP3 is located to the cell membrane where Cx43 can be found. When RIP3 is activated, the RIP1/3 complex will recruit MLKL to the lipid rafts of the cell membrane to execute necroptosis [316]. Likewise, connexins (Cx43, Cx32, Cx36, Cx46) are also targeted to lipid rafts when they are expressed [317]. This similarity in the locality could suggest that the RIP1/3 MLKL complex may be associated with Cx43.

4.3.7 Administration of Cx43asODN and clinical relevance of the study:

The upregulation of epidermal Cx43 was attenuated by the treatment with Cx43asODN. It could be possible that Cx43asODN downregulating Cx43 in other cell types of skin resulted in reduced inflammation [318]. For example, Cx43 could be downregulated in the endothelium, which resulted in reduced inflammation [319]. Cx43 could also be downregulated in dermal fibroblasts to prevent the spread of cell death, which would result in a less damaged dermis, leading to a mild PU [167]. The evidence indicates that Cx43asODN treatment is responsible for reducing I/R injury in the epidermis.

A single dose of Cx43asODN administered after ischaemic insult was effective in reducing PU progression. In treating a diabetic wound, a single dose was also used to elicit better re-epithelisation to kick start the healing process [151]. However, it remains unknown if administration before the insult or repeated doses would prove more effective. The design of a single dose was to a large extent influenced by the importance of Cx43 in normal skin homeostasis. Repeated administration could interfere with the normal functions of Cx43 if normal Cx43 expression is needed for skin homeostasis [147, 320].

It would be difficult to administer a drug to a patient as a prophylactic strategy without proper diagnosis and locational information of the PU. However, there are high-risk situations such as a surgical procedure where the patient could not be easily repositioned, prophylactic treatment could be administered at known danger areas such as the heel.

4.3.8 Conclusion

In conclusion, Cx43asODN treatment was able to prevent the increase of epidermal Cx43 and in turn, reduce I/R injury and the progression of experimental PU. This conclusion is supported by the observation that Cx43asODN treatment prevents epidermal Cx43 up-regulation, reduces neutrophil numbers and prevents the upregulation of epidermal HMGB1 in skin subjected to 1.5 h(I) / 4 h(R). Cx43asODN treatment was also able to prevent RIP3 upregulation after 1.5 h(I) / 4 h(R). This translated into the prevention of experimental PU progression in a double pinch I/R model when treated with Cx43asODN. Investigations into necroptosis suggest the possibility that Cx43 may be linked to RIP3 and HMGB1.

*Chapter 5. Characterisation of endothelial and neutrophil Connexin 43
knockout mice in ischaemia reperfusion injury*

5.1 Introduction

5.1.1 Cx43 in endothelial cells

Endothelial cells line the lumen of arteries, veins, and the lymphatic system to form a selectively permeable barrier between the lumen and surrounding tissues. These cells are vulnerable to injury in various diseases. In cardiac I/R injury, endothelial cells lose their anti-coagulant and anti-inflammatory properties, have impaired vasodilation, and show vessel hyperpermeability in response to lower pH, oxidative stress, and endothelial nitric oxide synthase (eNOS) inhibition [321-325].

During I/R injury of the heart, ROS is generated by cardiomyocytes, vascular endothelium and inflammatory cells [322]. In response, the endothelial cells become “activated” and precipitate intravascular micro-thrombosis, reduced blood flow, and further activate inflammatory cells [326]. Upon endothelial “activation”, E-selectin, P-selectin, and intercellular adhesion molecules (ICAMs) are expressed for neutrophils to recognise and bind to, facilitating extravasation into the injured tissue [326, 327]. Although endothelial cells are activated by ROS, more ROS is produced by them [324]. This occurs through xanthine oxidase, NADH/NADPH oxidase, and uncoupled eNOS, resulting in exacerbation of vascular dysfunction [324]. Concurrently after myocardial I/R injury, endothelial cells also lose barrier function, resulting in vascular hyperpermeability caused by ROS released from activated leukocytes [238].

Endothelial cells express connexins 37, 40 and 43 and all of them play different roles [328]. Endothelial cell-specific knockout of Cx43 (Cx43^{endo-/-}) in transgenic mice did not result in premature death [200]. However, the mice are affected by hypotension and exhibited bradycardia. These Cx43^{endo-/-} mice also exhibited increased plasma nitric oxide (NO), angiotensin (ANG) I and II [200]. These knockout studies demonstrated that although Cx43 is not essential for endothelial development, it is essential for the proper functioning of the vascular system. In contrast, Cx40^{-/-} mice showed hypertension attributed to irregular vasomotion [329].

Endothelial cells exposed to TNF α resulted in increased Cx43 perinuclear localisation in human umbilical vein endothelial cells (HUVECs) [330]. Similarly, expression of Cx43 was elevated when HUVECs were stimulated with IL-1 β and TNF α [331]. Furthermore, leukocyte adhesion and extravasation were reduced in animals lacking Cx43 expression in the endothelium [319]. These suggest the importance of endothelial Cx43 in regulating inflammation.

5.1.1 Cx43 in neutrophils

Connexins are known to participate in neutrophil-mediated inflammation. Cx43 was detected in hamster neutrophils after activation with lipopolysaccharide (LPS) [332]. This was confirmed by Brañes et al., showing that both Cx40 and Cx43 were expressed in human polymorphonuclear cells after stimulation with LPS or TNF α [333]. However, junctional communication between neutrophils was only active in the presence of endothelial factors secreted by endothelial cells when treated with LPS or TNF α [333]. The proposal that Cx40 and Cx43 were only expressed after activation

was challenged when Cx40 and Cx43 were shown to be present in unstimulated human neutrophils [334].

Treatment with Cx43asODN can prevent PU progression, but it was difficult to pinpoint how this was mediated. Stage 2 PUs have serum-filled blisters, which indicated oedema [29] consistent with vessel leakage and PU progression. Neutrophil numbers were found to be reduced in our study after Cx43asODN treatment (Fig. 4.4).

5.1.2 Hypothesis

I hypothesise that endothelial cell or neutrophil Cx43 plays a major role in the inflammatory processes in PU progression. If true, I/R injury would be expected to be moderated or reduced upon the knockout of Cx43 in either endothelial cells or neutrophils.

5.1.3 Aim

My aim is to investigate the role of both the endothelial cell and neutrophil Cx43 in PU progression. Endothelial specific inducible Cre recombinase transgenic mice or neutrophil specific Cre recombinase mice mated with Cx43^{flx/flx} mice were used to knockout Cx43 in endothelial cells or neutrophils *in vivo*.

5.2 Results

5.2.1 Genotyping of Cx43^{flx/flx}LysMCre mice

In order to address the question if Cx43 has any role to play in neutrophils, two Cx43^{flx/flx}LysMCre^{+/-} mice were mated (Fig. 5.1). All offspring were homozygous for Cx43^{flx/flx} gene as seen from the single PCR amplification product of 580 bp and a lack of the wild type PCR amplification product of 490 bp (Fig. 5.1a.). Four out of the eight offspring were heterozygous for the LysMCre gene (orange box, 700 bp and 350 bp product), two were LysMCre^{-/-} (green box, single 350 bp product), and two were homozygous for the LysMCre gene (red box, single 700 bp product) (Fig.5.1b, c).

Figure 5.1: Genotyping analysis of Cx43^{flx/flx}LysMCre^{+/-} mice progeny. According to information provided by The Jackson Laboratory, expected Cx43^{flx} product is 580 bp and wild type product is 490 bp. Expected LysMCre product is 700 bp and wild type is 350 bp. a) Mice were genotyped for Cx43^{flx}. Lane 3 is a 1 kb DNA ladder and lanes 1-2, 4-9 were loaded with DNA of Cx43^{flx/flx}LysMCre^{+/-} mouse progeny. Presence of a single 580 bp band and absence of 490 bp band indicates that mice are homozygous for Cx43^{flx}. b) Genotype of mice for LysMCre gene on a separate gel. Lane 1 is part of a 1 kb DNA ladder and lanes 2-9 were loaded with DNA of Cx43^{flx/flx}LysMCre^{+/-} mouse progeny. Presence of 700 bp band indicates that mice have at least 1 copy of the LysMCre gene. c) Genotype of mice showing bands for presence of wild type sequence on a separate gel. Lane 1 is part of a 1 kb DNA ladder and lanes 2-9 were loaded with the DNA of Cx43^{flx/flx}LysMCre^{+/-} mouse progeny. Presence of 350bp band indicates that mice have at least 1 copy of wild type sequence. Orange boxes indicate heterozygous mice having both LysMCre and the wild type sequence. Green boxes indicate homozygous wild type mice with no LysMCre gene. Red boxes indicate mice homozygous for LysMCre with no wild type gene sequence.

5.2.2 Cx43 is increased in neutrophils

After checking the genotype of the mice, neutrophils were harvested and purified from the blood of 7 – 12-week-old mice and stained for Ly6g (in red) and Cx43 (in green). The neutrophils were then analysed using ImageJ. Surprisingly, Cx43 staining was found on Cx43^{flx/flx}LysMCre^{+/-} and Cx43^{flx/flx}LysMCre^{+/+} neutrophils (Fig 5.2b, c). Furthermore, Cx43^{flx/flx}LysMCre^{+/+} neutrophils showed significantly more Cx43 (N=5, p<0.05) compared to their Cx43^{flx/flx} controls (Fig. 5.2d).

Figure 5.2. Cx43 is increased in neutrophils in Cx43^{flx/flx}LysMcre^{+/+} mice. Representative confocal images showing Ly6g staining in red and Cx43 staining in green of neutrophils extracted from a) Cx43^{flx/flx}, b) Cx43^{flx/flx}LysMcre^{+/-}, and c) Cx43^{flx/flx}LysMcre^{+/+} mouse blood. d) Graph shows quantified protein signal in neutrophils. Neutrophils extracted from the blood of Cx43^{flx/flx}LysMcre^{+/+} express significantly more Cx43 compared to the neutrophils of Cx43^{flx/flx} mice (N=5, p<0.05, Kruskal-Wallis, Dunn's post hoc test). Scale = 10 μ M

5.2.3 Genotyping of Cx43^{flx/flx}Tie2Cre mice

The crossing of Cx43^{flx/flx}Tie2Cre^{+/-} mice with Cx43^{flx/flx} mice, results in two genotypes Cx43^{flx/flx}Tie2Cre^{+/-} or Cx43^{flx/flx}. All offspring were found to be homozygous for Cx43^{flx/flx} demonstrated by the single amplification product of 580 bp and a lack of the wild type amplification product of 490 bp (Fig. 5.3a). Two offspring were genotyped and found to not have the Tie2Cre gene (red boxes, no 100 bp product) while six of the offspring expressed the Tie2Cre gene (100 bp product present).

Figure 5.3: Genotyping of Cx43^{flx/flx}Tie2Cre mice. According to information provided by The Jackson Laboratory, expected Cx43^{flx/flx} product is 580 bp and wild type product is 490 bp. Expected Tie2Cre product is 100 bp with accompanying internal control for amplification with product of 324 bp. Lane 1 is part of a 1 kb DNA ladder and lanes 2-9 were loaded with DNA of Cx43^{flx/flx}Tie2Cre^{+/-} mouse progeny. a) Genotype of mice showing bands for Cx43^{flx/flx}. Presence of 580 bp band and absence of 490 bp band, indicating that mice are homozygous for Cx43^{flx/flx}. b) Genotype of mice showing bands for presence of Tie2Cre. Presence of 324 bp band is an internal control that indicates proper amplification of gene target. Presence of 100 bp band indicates that mice have 1 copy of the Tie2Cre gene.

5.2.4 Connexin43 endothelial knockdown was confirmed by Cx43 immunostaining of endothelial cells of blood vessels harvested around the heart

After confirming the Tie2Cre gene is present in Cx43^{flx/flx} mice, tamoxifen was administered for 5 days, followed by a 7-day rest period to achieve knockdown of Cx43 in endothelial cells under the control of the Tie2Cre promoter. Endothelial cells in the blood vessel around the heart of Cx43^{flx/flx} mice with and without tamoxifen treatment were analysed and demonstrated to express normal Cx43 protein levels (Fig. 5.4a-f), while lower levels were observed in tamoxifen-treated Cx43^{flx/flx}Tie2Cre mice (Fig. 5.4g-i). Cx43 signal was quantified by image analysis and showed that there was ~55% reduction in Cx43 signal when comparing tamoxifen-treated Cx43^{flx/flx}Tie2Cre to Cx43^{flx/flx} blood vessels (Fig. 5.4j).

Figure 5.4: Cx43 is knocked down from endothelial cells. Representative confocal images of endothelial cells in the blood vessels connected to the heart from left to right of a) $Cx43^{flx/flx}$, b) $Cx43^{flx/flx}$ with tamoxifen and c) $Cx43^{flx/flx}Tie2Cre^{+/-}$ with tamoxifen with Cx43 stained in green. d) Quantified Cx43 protein signal showing Cx43 having a roughly 55% knock out. $N=3$, scale bar = $50\mu m$

5.2.5 Connexin43 endothelial knockdown does not prevent histological abnormalities of the skin after 1.5 h(I) / 24 h(R).

After 1.5 h(I) / 24 h(R) injury, similar observations were seen in insulted knockdown and control skin. These observations of the early tissue insult response (Fig. 3.5) included features such as spongiosis, hair follicle damage, loss of dermal cells, inflammation, and muscle degeneration (Fig. 5.5). Hair follicle damage appeared more severe while spongiosis seemed milder in both tamoxifen administered insulted Cx43^{flx/flx}Tie2Cre and Cx43^{flx/flx} skin when compared to previous observations (Fig. 5.5a-f). When comparing Cx43^{flx/flx}Tie2Cre and Cx43^{flx/flx} skin, there was no obvious rescue of the epidermis, hair follicles, inflammation, fibroblasts, and PC (Fig. 5.5).

Figure 5.5: Histological observations of skin at 1.5 h(I) / 24 h(R) of Cx43^{flx/flx} and Cx43^{flx/flx}Tie2^{Cre} mice. Panel of representative images. a-c) Both Cx43^{flx/flx} and Cx43^{flx/flx}Tie2^{Cre} mice exhibited spongiosis in their epidermis. d-f) Also, hair follicles show loss of sebaceous and follicular structures at 1.5 h(I) / 24 h(R). g-i) Leukocytes were observed in both mice. j-l) Dermis shows loss of fibroblast with inflammatory cells present in the centre of insult. m-o) Damage to the PC is also observed in both mice. N=5, Scale bar = 50µm

5.2.6 Connexin43 endothelial knockdown does not rescue vascular permeability

Previously, leakage was observed at 1.5 h(I) / 24 h(R) (Fig. 3.9). The link between endothelial Cx43 and vascular leakage was of interest and was investigated. The mean intensity of FITC-BSA was measured using the Zeiss Zen software provided with the Axioscan.Z1 slide scanner (Zeiss). At 1.5 h(I) / 24 h(R) of ischaemia, both Cx43^{flx/flx}Tie2Cre and Cx43^{flx/flx} skin with tamoxifen, showed FITC-BSA, which had leaked into the dermis (Fig. 5.6b, d). However, no apparent difference could be determined between Cx43^{flx/flx}Tie2Cre and Cx43^{flx/flx} skin (Fig. 5.6e).

Normal skin of $Cx43^{flx/flx}$ w Tamox 1.5 h(I) / 24 h(R) of $Cx43^{flx/flx}$ w Tamox

Normal skin of $Cx43^{flx/flx}Tie2cre^{+/-}$ 1.5 h(I) / 24 h(R) of $Cx43^{flx/flx}Tie2cre^{+/-}$ w Tamox

Figure 5.6: Vascular leakage at 1.5 h(I) / 24 h(R) of $Cx43^{flx/flx}$ and $Cx43^{flx/flx}Tie2Cre^{+/-}$ mice. Representative fluorescent images of FITC-BSA in green of a) normal skin and b) insulted skin of $Cx43^{flx/flx}$ mice and c) normal and d) insulted skin of $Cx43^{flx/flx}Tie2Cre$ mice e) Graph of quantified FITC-BSA mean fluorescent intensity and shows no difference in leakage in between both mice. N=4, Scale bar = 50 μ m

5.2.7 Connexin43 endothelial knockdown does not prevent up-regulation of epidermal

Cx43 at 1.5 h(I) / 24 h(R)

Figure 5.7: Epidermal Cx43 protein levels at 1.5 h(I) / 24 h(R) of $Cx43^{flx/flx}$ and $Cx43^{flx/flx}Tie2Cre$ with tamoxifen. Representative confocal pictures of a) normal and b) insulted skin from $Cx43^{flx/flx}$ mice and c) normal and d) insulted skin from $Cx43^{flx/flx}Tie2Cre$ with tamoxifen (Cx43 stained in green). e) Graph of quantified Cx43 protein showing Cx43 significantly increased in $Cx43^{flx/flx}$ mice (N=5, $p < 0.05$, Mann-Whitney U-test) increased but not significantly in $Cx43^{flx/flx}Tie2Cre$ mice (N=6, $p > 0.05$, $p < 0.05$, Mann-Whitney U-test). f) Graph of normalised epidermal Cx43 signal showing no difference in between $Cx43^{flx/flx}$ and $Cx43^{flx/flx}Tie2Cre$ ($p > 0.05$, N=6 $Cx43^{flx/flx}Tie2Cre^{+/-}$, N=5 $Cx43^{flx/flx}$ mice). Scale bar = 10 μ m.

Increase of epidermal Cx43 protein levels after 1.5 h(I) / 24 h(R) was observed and was of interest as a marker of I/R injury of the epidermis (Fig 3.11 and 3.12). At 1.5 h(I) / 24 h(R), the epidermal Cx43 increased in both tamoxifen administered insulted $Cx43^{flx/flx}Tie2Cre$ and $Cx43^{flx/flx}$ skin (Fig 5.7a-d). Cx43 in $Cx43^{flx/flx}$ mice was

significantly increased while epidermal Cx43 of insulted Cx43^{flx/flx}Tie2Cre skin only slightly increased (Fig 5.7e). Cx43 up-regulation of insulted Cx43^{flx/flx}Tie2Cre and Cx43^{flx/flx} skin showed no difference (Fig 5.7f).

5.2.8 Connexin43 endothelial knockdown does not prevent double cycle pinch model

1.5 h(I) / 4 h(R) / 1.5 h(I) I/R injury progression

Figure 5.8: Cx43^{flx/flx} and Cx43^{flx/flx}Tie2Cre with tamoxifen mice show no difference in double cycle pinch model 1.5 h(I) / 4 h(R) / 1.5 h(I) I/R injury progression. Representative macroscopic images of Cx43^{flx/flx} and Cx43^{flx/flx}Tie2Cre with tamoxifen mice of day 1, 3 and 7 showing no obvious differences. (N=10, 5 Cx43^{flx/flx}Tie2Cre mice, N=8 Cx43^{flx/flx} mice)

To investigate if Cx43^{flx/flx}Tie2Cre has any impact on I/R injury progression, a double pinch model of 1.5 h(I) / 4 h(R) / 1.5 h(I) / 7 d(R) was performed. Macroscopic images showed no obvious differences in progression between tamoxifen administered insulted Cx43^{flx/flx}Tie2Cre and Cx43^{flx/flx} groups (Fig. 5.8).

5.3 Discussion

5.3.1 Summary of key findings

Mice with the genotype of 1) Cx43^{flx/flx}LysMCre and 2) Cx43^{flx/flx}Tie2Cre were successfully generated. 3) Despite having the relevant genes for Cx43 knockout in neutrophils, Cx43^{flx/flx}LysMCre heterozygous mice exhibited no signs of neutrophil Cx43 knockout (Fig. 5.2a-b, d). Instead, neutrophil Cx43 increased in Cx43^{flx/flx}LysMCre homozygous mice (Fig 5.2c-d). 4) Endothelial Cx43 was knocked down with a 55% efficiency in Cx43^{flx/flx}Tie2Cre heterozygous mice (Fig. 5.4). 5) However, endothelial Cx43 knockdown did not prevent vascular leakage or epidermal Cx43 upregulation (Fig. 5.5-5.7). 6) Endothelial Cx43 knockdown did not prevent PU progression in the double pinch model (Fig. 5.8).

The results will be discussed in two main parts. 1) Addressing the issue of unsuccessful Cx43 knockout in neutrophils. 2) The unsuccessful investigation of endothelial Cx43 knockdown in Cx43^{flx/flx}Tie2Cre^{+/-} mice.

5.3.2 The increase of neutrophil Cx43 in Cx43^{flx/flx}LysMCre^{+/+} knockout mice

Cx43^{flx/flx} transgenic mice have been used to specifically knock out Cx43 in the murine eye and endothelial cells [199, 200]. LysMCre mice have successfully been used to study the Stat3 gene in neutrophils [202]. As of yet, there is no literature detailing the mating of the two mouse genotypes to study Cx43 knockout in neutrophils. Mating both genotypes should result in the knockout of Cx43 in neutrophils. Cx43^{flx/flx}LysMCre^{+/-} and Cx43^{flx/flx}LysMCre^{+/+} mice were successfully generated (Fig. 5.1). To confirm the knockout, neutrophils were extracted from blood, purified, stained for Cx43, which was

quantified (Fig. 5.2). Unexpectedly, Cx43 expression was unchanged in neutrophils in Cx43^{flx/flx}LysMCre^{+/-} mice and, instead, increased in Cx43^{flx/flx}LysMCre^{+/+} homozygous mice.

No publications address increased Cx43 in neutrophils. However, publications have described what possible implications that it may cause. Eltzschig et. al. reported ATP release from activated neutrophils occurring via Cx43 channels by first showing dose-dependent inhibition of ATP release using 18 α -Glycyrrhetic acid [335]. This is further confirmed using Cx40 and Cx43 specific mimetic peptides. Only Cx43 specific mimetic peptide was able to elicit a concentration-dependant inhibition of ATP release from neutrophils [335]. Finally, Cx43^{-/-} mice also showed reduced ATP release [335]. Neutrophil adhesion to the endothelium is also mediated by ATP release. When ATP release is inhibited, more neutrophils adhere to the endothelium although the ability to transmigrate was not investigated [335]. In another publication, neutrophil recruitment to the inflamed lung showed that in Cx43^{+/-} mice, fewer neutrophils were recruited to the lungs while more neutrophils were recruited in Cx43^{K258stop/-} (Cx43 channels which cannot close) mice when compared to Cx43^{+/+} controls [336].

From our results, specific knockout of Cx43 in neutrophils using the LysM promoter resulted in increased Cx43 expression. Referring to the publication by Eltzschig et. al., there would be increased ATP release upon activation which would reduce adhesion to the endothelium [335]. Following this, there will be increased recruitment to the inflamed tissue as reported by Saredine et. al. [336].

5.3.2.1 Speculation: Cx43 playing a role in neutrophil development

Schneider et al. reported high LysMCre activity in neutrophils extracted from the lungs as early as E19.5 and maintained high activity at day 5 after birth [337]. However, neutrophil development occurs in the bone marrow and neutrophils in the lungs were mature neutrophils. According to a review by Lawrence et al., the development of neutrophils involves the differentiation from a myeloblast to a promyelocyte, to a metamyelocyte, and finally maturing into a neutrophil in the bone marrow (Fig. 5.9) [338]. Lysozyme is present in gelatinase granules, which coincides with the metamyelocyte stage, indicating that the LysM promoter could have started in the myelocyte stage (1 stage before metamyelocyte).

Cx43 is expressed in neutrophils [334]. Furthermore, Cx43 expression has been shown to increase upon LPS or TNF α stimulation [333]. In inflammation, neutrophil Cx43 hemichannels release ATP and this was suggested to be a central inflammatory response controlling endothelial function [339]. This opens the possibility that neutrophil Cx43 may also be important for other functions of the neutrophils. Could it be possible that Cx43 also plays a role in neutrophil development?

Figure 5.9: Diagram taken from Lawrence et al. (2018). Diagram describes neutrophil development and its associated granule, vesicle, and nuclear segmentation changes. Lysozyme is expressed at the metamyelocyte stage.

Speculation:

If Cx43 does play a role in neutrophil development from the myelocyte to metamyelocyte or metamyelocyte to neutrophil stage, no neutrophils should be observed in the event of 100% cre/lox recombination. However, neutrophils were observed with Cx43, suggesting these could be ones that did not have the Cre/lox recombination event and “escaped”. Although this may explain why neutrophil Cx43 was still observed in Cx43^{flx/flx}LysMCre^{+/-} mice, it does not answer the increase in neutrophil Cx43 from Cx43^{flx/flx}LysMCre^{+/+} mice.

5.3.2.2 Potential implication of Cx43 downregulators on neutrophil

Neutrophil development starts in the bone marrow and enters the bloodstream as a mature neutrophil [338]. This suggests that any administration of Cx43 down regulators would need to be concentrated at the bone marrow for it to affect neutrophil development if Cx43 does indeed play a role in neutrophil development.

According to Geary et al., antisense oligonucleotides with phosphorothioate backbones with ribose sugar modifications reached peak plasma concentrations within 3 to 4 h [340]. This modified antisense was found to be most concentrated at the liver followed by the kidney which is expected of organs responsible for clearance [340]. Other than the liver and kidney, the antisense oligonucleotides were found to be distributed to the bone marrow, adipocytes, and lymph nodes [340]. However, there was no report on the biodistribution of unmodified antisense oligonucleotides [341]. This could be due to the degradation of unmodified oligonucleotides in vivo [342, 343]. This is interesting as AF488-Cx43asODN was still observed in the skin after 1.5 h / 24 h I/R, both intracellularly and extracellularly (Fig 3.18). This could be due to Cx43asODN exhibiting a localised effect over a sustained amount of time as it was administered in 30% pluronic gel which slowly administers the treatment [253]. These publications suggest that while it is possible for modified antisense oligonucleotides to be distributed to the bone marrow, it is unlikely for unmodified antisense to do so. Thus, in the speculation that Cx43 plays a role in neutrophil development, the possibility of Cx43asODN exhibiting an effect is low.

5.3.3. Possible explanations for the unsuccessful investigation of endothelial Cx43 and its implications

5.3.3.1 Tamoxifen effects on inflammation

Knockout of endothelial Cx43 with 55% efficiency was observed in blood vessels around the heart (Fig. 5.4). However, this did not result in the prevention of I/R injury (Fig. 5.5, 5.6, 5.7, 5.8).

In these inducible conditional knockout mice consisting of Cre recombinase and a mutated human oestrogen receptor (Cre/ERT2), tamoxifen is needed for the Cre/lox recombination [203]. In a review about the effects of tamoxifen on immunity, evidence points to tamoxifen being immuno-modulatory and anti-inflammatory [344]. In a study on breast cancer cells, the observation that TNF α mRNA levels increased by activating the estrogenic receptor (ER) α were abolished by tamoxifen agonistic effect to ER α [345]. Tamoxifen is also to be able to prevent vascular dysfunction and reduce TNF α and IL-6 levels in ovariectomized rats [346].

In the I/R model, both TNF α and IL-6 mRNA levels were increased, and tamoxifen could complicate the injury progression by reducing TNF α and IL-6 mRNA levels (Fig. 3.16). Vascular dysfunction could also be prevented (Fig. 3.9). This could also explain why our Cx43^{flx/flx} with tamoxifen controls in the double pinch model showed reduced injury as compared to our double pinch in Chapter 3 (Fig. 3.13, 3.14, 5.8).

5.3.3.2 Tamoxifen, an intrinsic limitation to inducible models with inflammation involved

Tamoxifen, being both an inflammatory regulator and a necessary ingredient for inducible gene KO, creates a limitation. Furthermore, it is difficult to completely get rid of tamoxifen from the mice. According to a publication, tamoxifen has a half-life of 12 h in serum [347]. I allowed a week for tamoxifen to be eliminated, so there should be no significant amounts of tamoxifen left in the blood. However, tamoxifen and its metabolites were found to accumulate in tissues and were 8 to 70 fold higher than serum [348]. Reinert et al. also report that Cre/lox recombination continued to occur in pancreatic islets two weeks after administering tamoxifen in mice [349]. However, a recent publication by Wang et al. reports that tamoxifen does not affect ischaemia reperfusion renal injury in mice [350]. Dosages of 50, 100 and 200 mg/ kg/ day for five days was administered and there were no preventive effects on renal injury [350].

Tamoxifen can reduce inflammation and be retained in certain tissues but does not affect ischaemia reperfusion renal injury. This suggests that more studies must be done to confirm the effects of tamoxifen in the context of skin I/R injury. For example, test tamoxifen in the non-transgenic double pinch model described in Chapter 3 to see if tamoxifen exhibits any preventive effects on I/R skin injury with respect to inflammatory markers such as IL6, TNF α and MMP9. If tamoxifen has a preventive effect on I/R injury and its inflammatory markers, tamoxifen-mediated knockout mice models should not be used to study ischaemia reperfusion injuries.

However, specific Cx43 targeting is still important in pinpointing the exact cause of I/R which is what I intended to do. To circumvent this problem, other knockout mouse

models exist outside the use of cre/lox recombination to achieve a temporal specific knockout of the Cx43. For example, clustered regularly interspaced short palindromic repeats (CRISPR)/Cas9 gene-editing technology could be used to achieve tissue specific knockout of the Cx43. A publication by Hagar et. al. managed to achieve tissue-specific CRISPR/Cas9 gene knockout in *Drosophila* by mating flies expressing tissue specific Cas9 with flies expressing the guide RNA (gRNA). Cells with both elements of activated Cas9 with gRNA will achieve tissue-specific knockout of the target gene [351]. This is also possible with Cx43 as human Cx43 has been shown to be knocked down using CRISPR/Cas9 technology in-vitro using HEK293 cells and is comparable to the knock-down using shRNA [352].

5.3.3.3 Connexin43 endothelial knockdown does not rescue vascular permeability

Several studies have shown that blocking Cx43 reduced vascular permeability and increasing Cx43 increase vascular permeability. Zhang et. al. reports that reduced Cx43 expression using Cx43 RNAi prevented vascular permeability in-vitro using pulmonary vein vascular endothelial cells treated with LPS [353]. When Cx43 is overexpressed, vascular permeability increases without the need for LPS treatment [353]. Another publication by O'Donnell III et. al. also observed increased Cx43 in human pulmonary artery endothelial cells after a challenge with LPS the vascular permeability was attenuated with carbenoxolone, a gap junction inhibitor, and siRNA of Cx43 [354]. Additionally, Danesh-Meyer et al. showed that vascular permeability in rat retina following ischaemia reperfusion was reduced when treated with Cx43 mimetic peptide [176]. The above studies suggest that vascular permeability was expected to be reduced in our Cx43^{flx/flx}Tie2Cre mice. However, vascular permeability was found to be no

different between the Cx43^{flx/flx} and Cx43^{flx/flx}Tie2Cre mice. As discussed above, the possible implication of tamoxifen could be the anti-inflammatory effect that may have resulted in reduced vascular permeability in both mice genotypes. There is also a possibility that endothelial-specific Cx43 knockdown does not attenuate vascular permeability in the context of an ischaemia reperfusion injury.

5.3.4 Conclusion

The observation of Cx43 up-regulation in neutrophils of Cx43^{flx/flx}LysMCre^{+/+} is unexplainable but steps such as sequencing could confirm the genotype. As Cx43 was not knocked out, no meaningful experiments could be performed on the mice magnet pinch model with regards to the effect of neutrophil Cx43 knockout in I/R progression unless the increase in Cx43 is explained. Furthermore, the study exposes the possibility that Cx43 may play a role in neutrophil development.

From our results, more experiments need to be done to evaluate the effects of tamoxifen before studying endothelial Cx43 knockdown. This is due to the possibility that tamoxifen is still present in the skin and could exert anti-inflammatory effects. While it is also possible that endothelial Cx43 plays no role in I/R in the skin, this remains to be confirmed.

Chapter 6. Future work

This chapter will address additional observations that were not expanded upon and directions forward in continuation to this study.

6.1 Summary

In this whole study, my hypothesis revolves around I/R mice model generation and looking at the early processes of I/R injury such as Cx43 upregulation, inflammation, and vessel hyperpermeability. I managed to generate a mouse model which allowed for the characterization of early processes which is an indication of early I/R injury. In this mouse model, I was able to identify increased epidermal Cx43, vessel permeability, loss of dermal cells, inflammation, PC damage. The mouse skin is still intact and did not progress into a wound, further supporting that these observations do not result in a Stage 2 PU (Chapter 3) [355].

Next, I needed to develop a model to study whether Cx43 down-regulation resulted in the prevention of PU progression. I successfully generated a wound by applying a double pinch to the mouse skin and this model would allow me to test if Cx43asODN would prevent the progression from a Stage 1 PU to a Stage 2 PU in this model (Chapter 3).

With the established models, I characterised the effects of Cx43asODN on a single pinch and found that epidermal Cx43 was no longer elevated. Furthermore, necroptosis markers HMGB1 and RIP3 were also suppressed from upregulation. Histological analysis also suggested that there is reduced dermal and PCR damage in the treated

skin. These observations were further validated when Cx43asODN treatment did indeed reduce the progression of a Stage 1 PU to a Stage 2 PU (Chapter 4).

At this point, I have 1) established the longest possible ischaemia duration for the mouse model where the epidermis is still intact, 2) characterised the observations in an early PU such as elevated epidermal Cx43, inflammation, vessel hyperpermeability, epidermal spongiosis, PC damage, loss of dermal fibroblast, 3) developed a double pinch model, 4) characterised the double pinch model in terms of histological differences and inflammation, 5) establish that Cx43asODN was able to prevent I/R injury by suppressing HMGB1 and RIP3 expression. This suggests that my hypothesis that downregulating Cx43 prevents I/R injury by regulating necroptosis is true.

Next, I sought to identify the cell type in which Cx43 played a major role in I/R injury. As Cx43asODN was delivered subcutaneously, any of the cell types such as the epidermis, dermal fibroblast, endothelial cells, inflammatory cells, in the skin could be the cause for I/R injury. Taken together the availability of the transgenic animals in the lab, endothelial Cx43 and neutrophil Cx43 was studied. However, results suggest that both transgenic models are met with controversy which requires more experimentation. Thus the study that either endothelial Cx43 or neutrophil Cx43 playing a role in I/R injury was not complete.

6.2 Explore Cx43 in neutrophils

As observed in Chapter 5, Cx43 was not knocked out in neutrophils isolated from Cx43^{flx/flx}LysMCre^{+/-} mice. Surprisingly neutrophil Cx43 is increased in Cx43^{flx/flx}LysMCre^{+/+}. As discussed in Chapter 5, if the mouse has a functional

knockout neutrophil Cx43 gene, it would be interesting to study if Cx43 is involved in neutrophil development. Cx43 is important for the function of neutrophils in releasing ATP and swarming to the injury site [356]. However, there are many questions regarding the role of Cx43 in neutrophils. 1) Cx43 has been confirmed to be expressed in neutrophils but it is not clear at which stage during its development neutrophil Cx43 gets expressed. 2) Cx43 half-life in neutrophils is not known. As reviewed by Berthoud et al., the current Cx43 half-life in various cells is less than 3 hours but it also shows that different cell types can have different Cx43 half-lives [357].

6.3 Exploring dermal Cx43 involvement.

Throughout the study, only the epidermis was studied for Cx43 expression and changes. Work done in past PhD preliminary studies pointed to an increase in dermal Cx43 in the insulted region. Taken together with publications citing an increase in Cx43 expression after I/R insult, future studies could include dermal cells such as fibroblasts [159, 160]. However, during I/R, many leukocytes migrate to the insulted area and since neutrophils and even macrophages are capable of expressing Cx43, more attention should be given as to which Cx43 expressing cells are contributing to the problem [358].

6.4 Exploring the epidermis thickening effect.

Mouse epidermis was found to have developed multiple layers after 1.5 h(I) / 4 days (R) at the edge of insult and 1.5 h (I) / 8 days (R) at the centre of insult as compared to its 2 to 3 cell thick epidermis. This is reminiscent of human skin which has layers such as the stratum basale, stratum spinosum, stratum granulosum, stratum lucidum and

stratum corneum. In figure 3.7, the epidermis at the edge shows that cuboidal cells are lining the base. On top of the columnar cells are what seems to be the stratum spinosum followed by the stratum granulosum and finally stratum corneum. This could be potentially useful for research that is interested in the multi-layers of the skin such as psoriasis and eczema. However, this remains to be confirmed by epidermal markers such as involucrin, CK1, CK14, Collagen IV.

Figure 6.1: Thickened epidermis at the edge of 1.5 h(I) / 4 d(R) I/R insult. Epidermis is hyper-thickened but different keratinocyte morphology can be seen. a) Terminally differentiated keratinocytes called corneocytes with nucleus almost removed. b) Keratinocyte layer appearing to be granular. c) Keratinocytes with a polyhedral morphology. d) Keratinocyte layer with columnar morphology.

6.5 Establishing hemichannel or Gap junction communication.

Our study uses Cx43asODN to downregulate Cx43 and does not tease out if it is reduced hemichannel activity or reduced gap junction communication. Mimetic peptides such as Gap19 can be used specifically to inhibit Cx43 hemichannels without disrupting gap junction communication temporarily [173]. This is important as neutrophils can release ATP via Cx43 hemichannels [356]. Furthermore, Cx43

hemichannels initiate and propagate calcium alarm signals leading to dense neutrophil swarms [335].

6.6 Exploring Necroptosis pathway with regards to Cx43

As discussed in chapter 4, Cx43asODN reduces the expression of RIP3 protein, a crucial protein for necroptosis. Yuan et al. reported that using Gap26, a Cx43 hemichannel and gap junction blocker prevents acute kidney injury caused by hypoxia-reoxygenation [315]. They reported that the injury was prevented by regulating reactive oxygen species by inhibiting Cx43 [315]. However, it is interesting that blocking Cx43 using Gap26 would also result in a lower RIP1 expression compared to the control [315]. Our data also shows that Cx43asODN treatment resulted in a lower expression of epidermal RIP3 (Fig 4.9). The results taken together could be pointing to Cx43 regulating necroptosis by interactions with RIP1 and RIP3 and would open another function or binding partner of Cx43.

6.7 Establishing the impact of cell specific Cx43 in I/R injury

During the study, endothelial Cx43 and neutrophil Cx43 was tested but no conclusion can be made. Future work including epidermal Cx43, dermal fibroblast Cx43 and macrophage Cx43 could also be tested to find out if any major cell types are contributing to ischaemia reperfusion injury. Furthermore, it could lead to the discovery of the interacting cell types (neutrophils – endothelial cells, neutrophils – dermal cells, etc.) that are the cause of ischaemia reperfusion injury.

6.8 Scarring

The double pinch model could be used to study scarring effects caused by ischaemia reperfusion. Less scarring could reduce the risk of a PU developing again in the same area by promoting better skin healing. A report based on recurring pressure ulcers on persons with spinal cord injury observed a 63% recurrence rate at the same location, suggesting that the skin may not have healed properly [359]. For scarring studies, ECM markers such as the collagen I to III ratio is important [64]. To test if Cx43asODN has preventive effects on recurrence, a double pinch can be done and allowed to heal for several days followed by another single pinch to compare if treatment will prevent a recurrence of the pressure ulcer.

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