Validating the relationship between burn temperature, duration of exposure and tissue injury severity for scald burns

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Faculty of Medicine
Abstract

Introduction:

Scald injury events are a common occurrence in early childhood. To reduce the risk to children of sustaining severe burn injuries, understanding the relationship between water temperatures, duration of exposure and tissue injury severity is essential. Current data used to predict the severity of burn injury and guide global scald burn prevention strategies is from the 1940s and has never been validated histologically for differentiating between superficial and deep dermal damage. While all burn injuries are painful and distressing for a child, severe deep dermal burns will require hospitalisation for extensive medical treatment, surgical intervention and may lead to scarring in the long term. Therefore it is essential to appreciate not only the burn conditions predicted to result in a cutaneous injury, but also to estimate the severity of injury, with particular reference to the more serious and clinically relevant deep dermal and full thickness burns.

Thesis aim:

The primary aim of this research was to review the current field of burn injury prediction and obtain evidence for the burn conditions required to cause severe deep dermal scald injuries. An appropriate animal model was used, with both histological and clinically relevant assessments of tissue injury severity.

Methodology and Results:

Systematic review: Forty-two studies were included in a review of porcine burn models where depth of burn was evaluated histologically. The mechanism of burn injury e.g. scald or contact with hot metal, was shown to affect injury severity results. Additionally, a gap in knowledge was identified regarding predicting the severity of injury to the dermis from exposure to moderate temperature water (50–70°C), as seen with hot tap water immersion scalds.

Experimental animal studies: A porcine scald model was successfully developed and optimised to examine the range of burn conditions representative of spill/splash and immersion scald injury events similar to those encountered by children. In total, 20 burn combinations were tested including 50 to 60°C water for 1 to 10 minutes (immersion) and 60 to 100°C water for 5 seconds (spill/splash). Wound examination, histological assessments and blood perfusion to the wound were analysed in the acute post-burn period.
(7 days). The burn conditions to result in mid-to-deep dermal injury were identified and included: 50°C for 10 minutes, 55°C for 2 minutes, 60°C for 30 seconds, and ≥ 75°C for 5 seconds. To evaluate the relationship between histologically determined tissue injury severity and the clinically relevant outcome of time to complete wound re-epithelialisation for these moderate to severe burns, a subset of 10 burn combinations were followed for 21 days. Damage to ≥ 75% of the depth of dermis was associated with burns taking longer than 3 weeks to fully re-epithelialise. Burns which were not fully re-epithelialised by day 21 included: 50°C for > 10 minutes, 55°C for 5 minutes, 60°C for 60 seconds, 70°C for > 15 seconds, and 85°C and 90°C for 5 seconds.

Mathematical modelling: Temperature changes within the skin during experimental scalding were recorded and used to develop a mathematical model to examine the thermal properties of skin. Overall, the thermal diffusivity (α) was estimated to be $0.03 \pm 0.02$ mm$^2$/s, and was independent of the burn duration, burn temperature, and skin thickness.

Clinical translation: A retrospective database review of Queensland’s major paediatric burns referral centre was conducted to identify and characterise the occurrence of severe scald burns in children. Forty-three cases of severe scald injuries which required surgical treatment ((split-thickness skin grafting (SSG)) were identified. The majority of SSG cases arose from a spill/splash event, with hot beverages (n = 17) and water from a saucepan/kettle (n = 14) the most common mechanisms. Freshly boiled water or recently made black tea/coffee were most likely to result in severe scalds.

Discussion:

The body of work presented in this thesis delivers compelling evidence for the first time to show that previous prediction data from the 1940s overestimates burn injury severity. Updated evidence-based estimates for the burn conditions (temperature and duration of exposure) to result in clinically relevant severe scald injuries are established and can be used to guide future global scald burn prevention strategies/legislation. This study also provided valuable, quantitative information about heat conduction in living skin with broad application to heat transfer modelling investigations of thermal injury prevention and thermal therapy studies.

Outcomes from this research project have already been used by burns clinicians in Queensland to inform police investigations where inflicted immersion scalds were suspected. This work also provided scientific evidence for regulators setting safety
standards in Australia for oven door temperatures.

There are three approaches to burn prevention: education of risk, safer physical design of products, and regulation and legislation. This research project provides valuable evidence-based scald injury prediction data to better inform these approaches and help keep children safe from burns.
Declaration by author

This thesis is composed of my original work, and contains no material previously published or written by another person except where due reference has been made in the text. I have clearly stated the contribution by others to jointly-authored works that I have included in my thesis.

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**Publications included in this thesis**

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**Publication 2 – incorporated as Chapter 3.**


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Contributions by others to the thesis

Dr. Leila Cuttle: conception and design of the project, assistance with interpretation of quantitative results, assistance with animal experiments and critical revision of thesis (early and final versions).

Prof. Roy Kimble: project design, critical revision of thesis (final version).

Prof. Mathew Simpson: conducted mathematical modelling and assisted with interpretation of research data for heat conduction presented in chapter 4.

Margit Kempf: assisted candidate with non-routine technical work to process samples and assistance with animal experiments.

Clay Winterford: assisted candidate with methodology and interpretation of histopathological staining techniques presented in Appendix A.

Dr Gael Philips: assisted candidate with development of methodology to identify and define pathological indicators of tissue damage in burned skin.

Statement of parts of the thesis submitted to qualify for the award of another degree

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Abbreviations

DDPT  Deep dermal partial thickness
°C  Degrees Celsius
°F  Degrees Fahrenheit
et al.  Et alia, and others
e.g.  For example
FT  Full thickness
H & E  Haematoxylin and Eosin
HBS  Hot beverage scalds
hr  Hour
IHC  Immunohistochemistry
LCCH  Lady Cilento Children’s Hospital
LDI  Laser Doppler Imaging
min  Minute
PLCBC  Pegg Leditschke Children’s Burns Centre
PU  Perfusion Units
QLD  Queensland
QPBR  Queensland Paediatric Burns Registry
QUT  Queensland University of Technology
ROI  Region of interest
RBCH  Royal Brisbane Children’s Hospital
sec  Second
SSG  Split-thickness skin grafting
SD  Standard deviation
SEM  Standard error of the mean
SPPBC  Stuart Pegg Paediatric Burns Centre
SB  Superficial burn
SPT  Superficial partial thickness
TBSA  Total body surface area
UQ  University of Queensland
Chapter 1  Introduction and literature review

1.1  Chapter foreword

This chapter provides a brief introduction which summarises the motivation for this research project. The literature review includes an overview of burn injuries, providing a background level of understanding with regard to burn depth/severity evaluation and burn wound healing. The strengths and weaknesses of previous studies in this field are examined and key knowledge gaps regarding injury prediction data for scald burns are identified. Finally, the aims of this research project and an outline of the thesis structure are described.
1.2 Introduction

Worldwide the frequency of serious scald burn injuries remains high, particularly for young children. To aid scald burn prevention strategies designed to avoid or reduce the risk of a serious burn injury, it is essential to have valid injury prediction data. This data is also necessary for counselling regulators setting thermal injury safety standards and to inform clinicians providing medicolegal judgements for scald injury litigation or where an inflicted injury is suspected. Despite the importance globally of preventing scald burns, evidence-based injury prediction data is lacking for the burn conditions (water temperature and duration of exposure) likely to result in a severe deep burn rather than a more superficial injury. This research project aims to address this deficiency by validating the relationship between burn temperature, duration of exposure and tissue injury severity.

Exploring the relationship between temperature, duration of exposure and the resultant injury severity is not a new concept. In the 1940s pioneering work by Moritz and Henriques established that the higher the water temperature, the shorter the exposure time required to produce a full thickness burn (1). Data from this seminal study established a time and temperature threshold for onset of cutaneous injury (irreversible epidermal damage) in humans and forms the basis for guidelines for our global approach to scald burn prevention to the current day. However, Moritz and Henriques study failed to consider histological verification of depth of injury to the dermis and this omission creates doubt over the validity of their results for deep dermal injuries.

A key difference between the current day approach to burn treatment management and that of the 1940s (when Moritz and Henriques were investigating scald burns) is that of early surgical intervention for deep dermal partial thickness (DDPT) and full thickness (FT) burns. Burns which heal within two to three weeks usually do not scar whereas deeper dermal burns, which take longer than 3 weeks to heal, almost always result in a scar (2-8) and may require grafting and arduous long-term management. Therefore, increased emphasis is now placed on early, reliable and accurate identification of burn severity, especially differentiating superficial partial thickness (SPT) and DDPT injury. However, it remains unclear what burn conditions are more likely to result in a severe scald injury (which will require surgical intervention and have an increased likelihood of scarring) rather than a more superficial injury.
This introductory section has provided only a brief overview of the motivation for this research project. The literature review will now go on to describe in greater detail the importance of this research and further identify key knowledge gaps regarding scald burns. Additionally, the strengths and weaknesses of the methodological approaches used by previous studies is evaluated.

1.3 Literature review

1.3.1 Paediatric burns

Burns remain a common and potentially devastating cause of injury in childhood. Severe burns often require extensive surgical intervention and long-term scar management therapy. Burns such as these are not only painful and traumatic for the child (9), but also place a significant burden on their families and health services (10). According to a review by Rayner and Prentice (10), the 0–4 year old age group has the highest incidence of burn injuries worldwide. Their review also concluded that globally, in regards to mechanism of thermal injury, scalds are the most commonly treated paediatric burn (10). This finding is supported by Australian National Injury Surveillance (ANIS) data which reports that of the 2000 children hospitalised each year in Australia as a result of thermal injuries (11), more than half of the admissions were for scalds (exposure to hot liquid or steam) (12, 13). Further, the total number of children with burns severe enough to present to physicians is likely to be much higher than the ANIS figures suggest, with a recent study (14) suggesting a ratio of outpatient to inpatient presentations for paediatric burn cases of 3.2:1. Similarly, Stockton et al. (15) argue that a more accurate representation of paediatric burn injury is obtained by including all presentations to hospital, reporting that only 12.7% of the children presenting for a burn injury to the Royal Brisbane Children’s Hospital (RBCH) in 2013 were initially treated as inpatients. Regardless of the exact numbers, it is clear that thermal injuries and in particular scald burns are an important paediatric public health issue.

A child can sustain a scald injury through a variety of mechanisms. Most commonly scald injuries occur as a result of a ‘spill’ or splash’ involving very hot water e.g. hot beverage scalds, water from kettles and cooking pots. Hot beverage scalds (HBS) have been identified as a leading cause of scald injuries in young children (16, 17); in an Australian hospital setting (17) HBS were reported to account for one in five of all burns treated. However, scalds can also result from accidental or non-accidental ‘immersion’ in more moderate temperature water, such as hot tap water, for relatively longer durations (16, 18, 19). In their
review of inflicted scalds in children, Maguire et al. (19) determined that compared to other
types of burn injuries, hot tap water scalds were more likely to represent child abuse. Given
the wide range of mechanisms and burn conditions described which can result in a child
sustaining a scald injury, understanding the relationship between water temperature,
duration of exposure and tissue injury is essential for guiding scald prevention approaches.

1.3.2 Classification of burn injury severity

1.3.2.1 Anatomy of burn wounds

Before proceeding to review the research regarding prediction of scald injury severity, it is
necessary to understand the structural anatomy of the skin (Figure 1-1). The epidermis is
the thin outer protective layer of the skin which consists mainly of epithelial cells and keratin.
The dermis, which is much thicker than the epidermis, is composed of collagen and elastin
fibres, and contains skin appendages, with vascular supply to the skin and nerves (20). The
dermis is further divided into two layers: the upper, narrow papillary dermis which contains
the numerous small vessels of the superficial sub-papillary vascular plexus; and deep to
this, the relatively thicker reticular dermis which contains the larger vessels of the deep
vascular plexus, the hair follicles and glandular structures. Below the dermis is the fatty
tissue of the subcutis.

Figure 1-1 Anatomy of the skin. A three-dimensional illustration of the skin showing the depth of burn.
Adapted from Shupp et al. (21)
It is of particular importance to note that the appendages (hair follicles, sebaceous and sweat glands), located deep in the reticular dermis, act as an important reservoir of regenerative stem cells for keratinocytes (22). If these appendages are undamaged by thermal insult, there is an ample supply of epithelial cells to repopulate the epidermis and wound healing is more likely to be successful. Conversely, if the depth or severity of the burn is such that the majority of the appendages are destroyed, then re-epithelialisation is effectively restricted to the edges of the wound and is more likely to be prolonged which may necessitate surgical intervention (22).

Recognition that the depth of a burn has a significant influence on the potential for successful wound healing is crucial to clinical decisions regarding how a burn should be treated. However for most burns, especially scalds, the depth of an individual burn is unlikely to be uniform i.e. there is a tendency for burns to be deepest at their centre and shallower at their periphery (23). In 1953, Jackson (23) was the first to describe the appearance of three concentric zones of a burn: a central area of irreversible tissue loss with complete coagulation and denaturing of proteins; an intermediate area with compromised vascular perfusion; and a peripheral area characterized by vasodilation and inflammatory changes, but no structural damage. Injured cells in the intermediate zone which may have survived the initial thermal insult may either recover or progress to become non-viable, causing a deepening of the burn wound over time i.e. burn wound progression. This dynamic phenomenon of burn wound progression is of particular importance for partial thickness burns where an initial SPT or DDPT burn may progress over a period of 2–4 days to a DDPT or FT burn (3, 24).

### 1.3.2.2 Classifications used to describe burn injuries

When reviewing the early historical literature discussing classification of burns (23, 25, 26), contention surrounding the optimal terminology useful for describing the severity of a burn wound is apparent. Historically, burn wounds were described by degrees from first to fourth based on their severity (25). However, by the 1950s it was recognised by Jackson (23) that classifications based on the surface appearance or ‘intensity’ of the burn may be misleading and proposed that it may be of more appropriate to recognise the depth of a burn, which he broadly classified into two categories; partial thickness and full thickness skin loss. It is now well accepted that classification of burn wounds based on the depth of the thickness of the damaged skin is superior and should be considered standard (3, 7, 21, 27-30). For research purposes this classification is most commonly described (21, 31) as presented in Table 1-1.
Table 1-1 Classification of burn wounds

<table>
<thead>
<tr>
<th>Burn Classification</th>
<th>Abbreviation</th>
<th>Description</th>
<th>Previous classification</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>SB</td>
<td>Affects only the epidermis</td>
<td>1°</td>
</tr>
<tr>
<td>Superficial partial thickness</td>
<td>SPT</td>
<td>Extends through the epidermis and into the papillary dermis</td>
<td>Shallow 2°</td>
</tr>
<tr>
<td>Deep dermal partial thickness</td>
<td>DDPT</td>
<td>Involves the reticular dermis (deeper layer of dermis)</td>
<td>Deep 2°</td>
</tr>
<tr>
<td>Full thickness</td>
<td>FT</td>
<td>Involves whole thickness of dermis, may extend into subcutaneous tissue</td>
<td>3°</td>
</tr>
<tr>
<td>Full thickness, involvement of underlying tissues</td>
<td></td>
<td>Extends through subcutaneous tissue, may involve muscles or bones</td>
<td>4°</td>
</tr>
</tbody>
</table>

With dramatic advances in surgical techniques and treatments for burns over the last four decades, the usefulness of the terminology describing degrees or SPT or DDPT in a clinical setting has been questioned (7, 29, 30). A recent review of burn depth assessment by Monstrey et al. (7) contends that, in a clinical setting, precise quantification of burn depth may be of a more theoretical value. The review suggests that from a clinician’s perspective a more useful division may be to classify burns into ‘superficial’ wounds that heal by conservative treatment versus ‘deep’ wounds that would require surgical therapy. It is important to be aware of these issues when considering the translation of research results to a global health care setting.

1.3.3 Experimental studies investigating the time and temperature threshold for burn injuries

Thermal injury studies conducted in the 1940s by Moritz and Henriques (1, 32, 33) are arguably the most influential and frequently cited papers examining the relationship between tissue injury depths, duration of exposure and temperature. Prior to the studies of Moritz and Henriques, limited investigations regarding the time and temperature threshold for skin damage had been presented by Hudack and McMaster (34), and Leach et al. (35) who reported on experiments conducted using mice and guinea-pigs respectively. However, it was Moritz and Henriques who were the first to establish a time and temperature threshold for cutaneous injury in humans. By first creating a comprehensive dataset utilising a porcine scald burn model, Moritz and Henriques were able to investigate a much smaller number of
critical exposure values (33 exposures) on a modest number (eight) of human volunteers. They concluded that human and porcine skin was similarly susceptible to thermal injury (1) (Figure 1-2). Results from this study have been universally accepted and form the basis for burn prevention strategies and legislation regarding safety guidelines for hot tap water delivery worldwide.

Figure 1-2 Duration of exposure vs temperature and severity of tissue damage in porcine and adult human skin. A. Porcine skin; B. Adult human skin. Adapted from Moritz & Henriques 1947(1).

Although validating depth of injury with histological analysis is widely accepted as gold standard, a major limitation of Moritz and Henriques study (1) was that no biopsies were taken from the burns they created in human skin. Entin and Baxter (36) noted this deficiency and sought to further test the validity of the findings in man by conducting experiments on humans for a range of temperatures (50–110°C) and exposure times (3, 5, 10, 15 seconds) with a hot metal disc burning apparatus. Whilst this data further enhanced understanding of experimental burns in normal human skin and sought to correlate the transition of clinical and microscopic changes after a thermal injury (36), due to inherent ethical issues surrounding research on humans, they were limited by a small sample size and modest single burn areas. Additionally, Entin and Baxter’s data was created using contact with metal as the mechanism of injury, rather than a scald injury. Understandably, since that time, there have been no similar human experimental burn models published. All modern in vivo experimental work in this area has been carried out in various animal models, using a variety of apparatus to create a range of thermal injuries (2, 37-47). The various strengths and weaknesses of the different animal models for thermal injury investigations is discussed in the next section of this chapter.

Experiments with human subjects conducted by Moritz and Henriques (1) relied only upon
external (macroscopic) appearance of the burns created as an indicator of depth, without verifying this with histology. The significance of this limitation is highlighted by the clinical work on human subjects by Jackson (23) who documented the value of the physical appearance of burns with respect to early diagnosis of the depth of injury. Jackson clearly demonstrated that classification of burns based solely on their surface appearance (intensity) can be misleading. Instead, Jackson emphasized the importance of focusing upon the depth of tissue destruction for diagnosis of burn severity. Whilst the visual appearance of a burn is an invaluable tool to a burns clinician, it should not be considered the gold standard in a research setting.

Despite the prestige of their work, there are key weaknesses to the methodological approach taken by Moritz and Henriques (1) which are not well publicised. For example, their experimental design lacked standardisation for the number of replicates for each burn condition, limiting statistical analysis of results. In fact, although the inherent biological variability of burn wounds is well known, many of the burn conditions tested were not replicated. Additionally, it is unclear from the manuscript whether investigators were blinded to the burn conditions being tested. While it is difficult to apply today’s standards to a study which was completed over 70 years ago, the methodologic weaknesses described are still a valid concern as data from this study continues to be used as a reference standard for current day scald burn research.

Unlike the experimental work with human skin, Moritz and Henriques porcine studies included some histological analysis (1). However, the method of histological analysis described has a number of serious limitations. Firstly, it is difficult to establish from the original paper (1) at what time points porcine biopsies were taken and what pathological changes they observed and used as criteria for identifying the depth of tissue damage. Secondly, the classification system used to define depth of burn was misleading and inaccurate. The responses Moritz and Henriques examined to thermal doses were limited to only one microscopic or pathological indicator; whether they had caused full-thickness destruction of the epidermis or not. All responses identified as having full-thickness epidermal damage were then further classified as either 2nd degree or 3rd degree burns based on unspecified qualitative assessment of ‘irreversible’ dermal injury. Therefore, while the time and temperature threshold curve for full-thickness epidermal damage is well described with histological evidence, the depth of damage to the dermis is not. Unfortunately, as noted by others (48, 49), the results from Moritz and Henriques thermal
studies have been and continue to be erroneously translated and extrapolated such that full thickness damage to the epidermis is incorrectly equated to a more severe full thickness burn.

There appears to be a paucity of relevant comprehensive experimental studies investigating the relationship between temperature, duration of exposure and tissue injury until the 1990s (37, 39, 40, 47, 50-53). Of these studies, Singer’s group (44, 54, 55) has published extensively and presented a comprehensive contemporary data set for a wide range of temperatures, durations of exposure and tissue injury depths in a porcine model. Making use of advances in histology and immunohistochemistry, they developed a more comprehensive and importantly, quantitative set of histological parameters to measure the depth of injury for specific cell types contained within the dermis. However, the burning apparatus used by Singer’s group (heated Aluminium bar) creates a contact burn rather than a scald burn. A difference in pathophysiology between scald and contact burns is speculated (37, 56), although there is a deficiency of studies published in this area. While water is a relatively poor thermal conductor compared to metals, it has a much higher specific heat capacity and therefore the amount of thermal energy stored in water heated to a certain temperature is greater than that of metals such as aluminium. Additionally, as previously described, scald injuries may be sustained via different mechanisms and it is unknown how the burn conditions established using a contact model may be more broadly applied to the range of scald injuries sustained by children.

In recent years, researchers in the burn injury field have focused on the creation/validation of predictable burn models in animals that can create a known, repeatable or consistent depth of injury (2, 46, 57, 58). Such models are essential for the assessment of healing efficacy for different therapeutics. Whilst data generated during the creation of these models (44, 54, 55, 57) provides a more accurate measure of the depth of injury to the dermis than Moritz and Henriques earlier work, they are less comprehensive with regard to the broad range of burn conditions tested. Additionally, the method most commonly employed for creating the thermal injury in porcine models is a contact burn via a heated metal comb or bar (46, 55, 57, 59-62) rather than a hot water scald. Quality experimental data is still lacking for the creation of burns using many different temperatures and durations in general and more specifically for hot liquid or scald burns. For example, currently there is no available data to provide good evidence to determine if a deep dermal burn was caused by an exposure to 90°C water for 5 seconds (consistent with a timeframe of an accidental burn) or
whether it was caused by an exposure to 70°C water for 20 seconds (more consistent with an inflicted injury mechanism). Examination of both shorter and longer durations of exposure than currently reported in the literature would provide valuable data to inform medicolegal judgements about accidental versus inflicted burn injuries in children.

1.3.4 Validity of different experimental models for burn injury prediction studies

1.3.4.1 Types of experimental burn models

Burns researchers have endeavoured to use both *in vivo* (animal) and *in vitro* models to study cutaneous thermal damage (44, 63, 64). A full literature review of experimental animals models used in burns research is beyond the scope of this report and is well summarized in a review by Abdullahi et al. (65). However, before proceeding to examine results for tissue injury severity, it is essential to understand the basic advantages and disadvantages of the relevant animal models and how these may affect the validity of the results obtained.

Substantial burn injury research work has been undertaken on mice and rats (41, 45, 47, 50, 51), primarily due to their relative ease of handling, the ability to use a large number of animals and their affordability (50, 63, 66). However, small mammals differ from humans in many important anatomical and physiological ways (67) (Table 1-2). The most significant differences relevant to thermal injury studies are small mammals (rats, mice and rabbits) have: a denser distribution of body hair (which undergoes a defined cycle of hair growth that is significantly different to humans (65)); a thinner epidermis and dermis; and most importantly a *panniculus carnosus* (a thin skeletal muscle layer) which causes wounds to heal primarily by contraction (68) (rather than re-epithelialisation, as in humans) and may also affect the way heat is conducted through the skin (67). These structural differences are an important consideration when assessing the translational relevance of using small mammal burn models.
Table 1-2 Comparative skin histology of mammals commonly used in burn research. Reproduced from Abdullahi et al. 2014 (65).

<table>
<thead>
<tr>
<th>Trait</th>
<th>Human</th>
<th>Pig</th>
<th>Rat</th>
<th>Mouse</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hair coat</td>
<td>Sparse</td>
<td>Sparse</td>
<td>Dense</td>
<td>Dense</td>
</tr>
<tr>
<td>Epidermis</td>
<td>Thick</td>
<td>Thick</td>
<td>Thin</td>
<td>Thin</td>
</tr>
<tr>
<td>Dermis</td>
<td>Thick</td>
<td>Thick</td>
<td>Thin</td>
<td>Thin</td>
</tr>
<tr>
<td>Panniculus carnosus</td>
<td>None</td>
<td>None</td>
<td>Present</td>
<td>Present</td>
</tr>
<tr>
<td>Skin architecture</td>
<td>Firmly attached</td>
<td>Firmly attached</td>
<td>Loose</td>
<td>Loose</td>
</tr>
<tr>
<td>Wound-healing mechanism</td>
<td>Re-epithelialisation</td>
<td>Re-epithelialisation</td>
<td>Contraction</td>
<td>Contraction</td>
</tr>
</tbody>
</table>

While no animal model is a perfect representation of humans, the advantages of using the domestic pig as a model for thermal injury studies are well documented (1, 65, 69, 70). In addition to having a sparse hair coat, similarities between human skin and pig skin include: a relatively thick epidermis with distinct rete ridges and dermal papillary bodies (42, 67); a comparable vascular supply to the hair follicles (42); and a similar distribution of blood vessels in the dermis (67). Importantly, like humans, pigs heal primarily by re-epithelialisation (67) as they have a similar collagenous tissue framework (42, 44) and do not have a panniculus carnosus muscle layer. Differences between human and pig skin should also be noted: pigs have an absence of eccrine (sweat glands), fewer elastin fibres in the dermis (44), more extensive fat deposited below their subcutis (69), and their subepidermal vascular network is less dense (44). In addition to having similar skin to humans, pigs also have the advantage of being a large enough size to allow multiple burns to be created on the same animal (44, 56) without resulting in a systemic stress response (44). Creating multiple burns on the same animal allows for the sample size to be increased, without increasing the number of animals used. The main disadvantage of the porcine model is that pigs are substantially more expensive to work with (65) and are more labour intensive from a husbandry aspect than small animals.

Substantial advances in cell culture techniques in recent decades have enabled the utilisation of in vitro models for thermal injury studies. However in vitro models such as cell
culture and skin explants are limited in their capacity to capture all aspects of burn pathophysiology (56, 65, 71, 72). The simplicity of a single cell culture model is better suited to investigating specific individual aspects of the burn injury process. Burn wounds are dynamic, involve multiple cell types and skin structures, and they can deepen over time. Therefore, studies investigating the acute and peri-acute effects of burn injury require a complex model. Models with a functioning vasculature system preserve the capacity to reproduce the elaborate local and systemic responses to thermal injury that occur in humans, such as stimulus of the coagulation and inflammatory cascades (71, 73). While in the future improved in vitro burn models more comparable to the in vivo situation may be developed, currently such a complex model, which allows systemic factors to be considered, is not available.

An alternative to both in vivo and in vitro models, which is well described in the literature, is using mathematical/computer models to simulate burn injury conditions and calculate the expected level of tissue injury (64, 74-79). Given the ethical considerations regarding the use of animal models for medical research and advances in technology, the use of mathematical modelling and simulations has increased. However, approaches of this kind carry with them various well known limitations, which will be discussed in more detail later in this chapter.

In summary, this section has reviewed the different types of experimental burn models available. Currently, the literature supports the acceptance of an in vivo porcine model as the thermal injury model of choice for studying the relationship between burn temperature, duration of exposure and tissue injury. Therefore, with regards to animal burn models, for the remainder of this thesis, emphasis is placed on reviewing relevant porcine burn models.

1.3.4.2 Mechanism of thermal injury- scald versus contact

There are many different mechanisms of thermal injury including: flame, contact with heated solids, scalds, lasers, chemicals and radiant heat exposure. Thus, there are also several different types of experimental burn models which use a broad range of methods and materials to create burns for investigation. The type of heat source chosen to inflict burns may influence the characteristics of the resultant burn due to differences in the mechanism and properties of heat transfer (80). For the purpose of validating a relevant temperature and time threshold for injury depth, the two main thermal insults cited in the literature are the scald burn model (1, 37, 38, 41, 45, 50, 81, 82) and the contact burn model (2, 36, 39,
40, 43, 44, 46, 47, 54, 83-88). The key advantage of contact models is the use of a simple single burning apparatus which poses minimal risk of injury to researchers (44). In contrast, it is often noted that creation of a scald burn is technically more challenging (44) and inherently poses a greater risk of thermal injury to investigators (2, 36, 44, 65). As a result, since the initial work done by Moritz and Henrique using a porcine scald model (1, 32, 33), relatively few published studies have used porcine scald models.

Clinically it is recognized that the pathophysiology of scald burns is different to that of contact burns (37, 56), however there is a notable paucity of information in the literature regarding this disparity. Brans et al. (37) conducted a small pilot study (one animal) using a porcine model to compare the histologic differences in tissue damage caused by scald burns as opposed to contact burns. Brans et al. (37) assert that contact burns cause an immediate coagulation, creating a clear line between viable and necrotic tissue on histological examination. They speculate that this necrotic layer may function as a barrier to heat conduction, reducing the damage to the deep vascular plexus. Conversely, they observed scald tissue damage to be comprised of an initial hyperaemia, followed by delayed vascular damage, causing a more intermingled pattern of collagen damage rather than a defined line (37). Interestingly, they also reported that for intermediate depth scald burns the superficial vasculature could still be intact even though the deep dermal plexus was damaged; as opposed to deep scald burns where both the superficial and deep vasculature structures were damaged. While the study of Brans et al. offers some insight into the differing microscopic appearance of scald burns compared to contact burns, the strength of the study is severely limited by having such a small sample size (only one replicate for each of 6 burn conditions) and further research in this area is necessary.

While there are no other porcine studies which compare the pathophysiology of scald burns to other mechanisms, in 2009 Tschop et al. published an experiment in mice comparing the effects of a scald burn versus a flame injury (82). Similar to Brans et al. (37), their data suggests that the scald model produces a more extensive injury and postulates this may be caused by an increased transfer of thermal energy resulting in increased tissue damage (82). Tschop's group used a subdermal probe to measure temperature changes in the skin and reported a significantly higher subdermal temperature at the end of the 9 second scald burn compared with the flame burn. Although this study describes the difference between a flame and scald model rather than a contact model of burn creation, it further supports the assertion that different types of burns may lead to different levels of vascular damage and
as such greatly influence the ultimate level of tissue damage (56).

1.3.4.3 Issues surrounding variability of burn creation in scald burn models

A major concern for studies using animal burn models is the challenge of creating reproducible burns of the same depth and severity. Fortunately some of the variables inherent to the contact burn model are negligible in the scald model e.g. uniform contact, adherence and heat capacity (80). However, there is scant discussion in the literature regarding how variations in burn technique may affect the reproducibility of scald burn models. For experimental scald models two main methods are described for providing direct exposure of hot water on to the surface of the skin. Firstly, for small mammals (mice, rats and guinea-pigs), investigators describe various templates designed to position anesthetised animals into a water bath in such a way that the area to be burned is immersed (41, 45, 50, 82, 89). Cribbs group (50) reports that when using this method an experienced technician may perform this procedure on 30–50 mice a day. Secondly, for large animals such as pigs, the method most commonly described involves placing an applicator filled with hot water pre-heated to a desired temperature directly onto the skin, e.g. a bottomless jug (37, 81, 86) or polypropylene tubes (38). A major limitation of this method is the inability to refresh and recirculate the water so that a consistent temperature can be maintained for the required duration. Since the thermal studies by Moritz and Henriques in the 1940s (1, 32, 33), there have been no porcine scald burn models described that adequately address the issue of maintaining a constant water temperature for the desired duration of exposure. Primarily, the focus for more recent studies has been to investigate burns created by exposure to very high temperature water for only a short duration (seconds), thus variability in water temperature over time is not identified as a significant issue. Nonetheless, Korompai’s group (85) reports (as an incidental observation) that upon removal from the heat source, boiling water decreased by 4ºC within only 10–15 seconds. For studies investigating the burn conditions involved in immersion scalds (moderate temperature water for long durations), methods to maintain consistency in water temperature are an important consideration.

1.3.5 How does the thickness of the skin affect susceptibility to thermal insult?

It is apparent from the literature both globally (90, 91) and in Australia (17, 92, 93) that children (< 5 years) and the elderly (> 75 years) are disproportionally affected by scald burns. Given the increased incidence of scalds in these age groups, it is important to consider how
variations in the thickness of the skin with age and or anatomical location may alter the susceptibility to thermal insult. It is commonly reported that skin thickness shows a gradual increase from birth to adulthood (94-96). Skin thickness can be measured in vivo using diagnostic modalities such as ultrasound (94, 97, 98) and measured ex vivo with histological analysis of biopsied skin (99-101). A comprehensive study by Seidenari et al. (94) used a 20MHz ultrasound to measure skin thickness and determined that thickness varied depending on both anatomical site and age: for children, thickness ranged from 0.86 to 1.48mm and for adults, from 0.98 to 2.34mm; with less thick skin in the lower limbs compared to the areas of the trunk and face. Although a reasonable amount of data exists, there is still some contention surrounding the exact definition of the effects of aging on whole skin thickness, which is beyond the scope of this review, but well summarised in a review by Walker et al. (102). Regardless, although conflict exists in the literature regarding the relative contributions that age, gender, ethnicity and body site have regarding variations in skin thickness, differences in skin thickness are apparent and may impact the validity of results extrapolated from an experimental burn model.

Differences between the susceptibility of a child’s skin versus an adult’s skin to thermal insult is reflected in the current tap water safety legislation (The Plumbing Code of Australia (PCA), Australian and New Zealand Standards 3500 series and the Water and Sewerage Act 2001) which mandates that hot water from bathroom taps be delivered at no greater than 50ºC for most dwellings and not exceeding 45ºC for early childhood centres, schools and nursing homes. This legislation is based on the work of Moritz and Henriques (1). As explained earlier in this chapter, Moritz and Henriques observed superficial injuries in adult humans, then extrapolated (from porcine experimental data) for more severe injuries. Safe water temperatures for children have then been inferred from these existing adult data (96, 103). Concerns regarding the adequacy of hot water temperature safety legislation (introduced in the 1980s in USA) in relation to children were first raised in 1983 by Feldman (96). Using Moritz and Henriques original mathematical equations (1, 32), Feldman calculated the critical thermal injury threshold times for children based on adjustments made for their thinner skin (Figure 1-3). Feldman asserts that at temperatures of greater than 54ºC, children can burn in about one fourth of the time of adults (96).
Figure 1-3 Critical threshold temperature for scald burns in children versus adults for first degree and full thickness burns. Adapted from Feldman 1983(96).

In the grey literature regarding guidelines for prevention of scalds in children e.g. Kidsafe Australia, World Health Organisation (WHO), International Society for Burn Injuries (ISBI)), it is routinely asserted that children’s skin is more susceptible to thermal injury than that of adults. However, published scientific evidence to support this assumption is less readily available. Discussion in the literature regarding the increased susceptibility to thermal injury of a child’s skin compared to an adult’s skin centre around the contention that a child’s skin is thinner than that of an adult (94-96, 103-106). It is proposed that thinner skin is more vulnerable to scald injury because heat penetration is proportionate to skin thickness (96) i.e. if a thermal insult is applied to the surface of thinner skin it will cause damage to a larger fraction of the total skin thickness (95). This is supported by mathematical modelling (77) where epidermal and dermal thickness were found to have a direct impact on the temperature distribution within the skin layers. Others, such as Diller (95), have also used mathematical modelling (adapting Moritz and Henriques model (1, 32)) to predict how thermal injury to a child compares to an adult, however, no experimental data is presented to validate these results. It remains difficult to estimate how differences in the skin thickness (with age and/or anatomical location) may affect the susceptibility of the skin to scalding. Further knowledge in the area is essential for guiding any future global recommendations regarding the setting of standards for hot water safety.
1.3.6 Mathematical modelling to estimate burn injury severity

It is well recognized that the creation of a mathematical model which may be used to predict the depth and severity of scald burns which result from certain temperature and time conditions would be highly advantageous. Injury prediction models such as these have broad applications both clinically and for burn prevention. Ideally, this would enable clinicians to predict the depth of a burn injury without having to rely solely upon visual observation or invasive diagnostic tools. Several attempts to create a workable mathematical model are described (64, 78, 79, 107-109) and will be reviewed here. As with the experimental scald modelling, much of the early work in this area was pioneered by Moritz and Henriques with their studies of heat conductivity and thermal injury (32).

Moritz and Henriques data showed an inverse relationship between temperature and the time taken to produce a certain severity of thermal injury (32). They developed an analytical model applying an Arrhenius equation to predict thermal damage and derived a damage function, $\Omega$ (see Figure 1-4) which correlated with experimental data from their porcine model (32). The coefficients determined by Moritz and Henriques were; $\Omega = 0.53$ for a first degree burn; $\Omega = 1.0$ for a second degree burn; $\Omega = 10^4$ for a third degree burn. These coefficients have been widely applied for the last 50 years by many investigators and continue to be used by current researchers such as Ng et al. (76), Abraham et al. (64) and Bellia et al. (77). While Moritz and Henriques mathematical model is noteworthy, unfortunately the same limitations discussed for the experimental model, namely lack of histological evidence for describing the onset of dermal injury for second and third degree burns, also apply here.

$$\Omega(x, y) = \int_0^t A \exp \left[ \frac{-\Delta E}{RT(x, y, t)} \right] dt$$

Figure 1-4 Equation to estimate the severity of burn injury as a function of skin depth and radial position. Reproduced from Diller and Hayes (107)

It was not until the 1980s, when investigators were able to make use of significant advances in computing capacity to develop finite element models (75, 107, 108), that the effect of blood perfusion during burn injury was able to be taken into account. Diller, Hayes and Blake (75) were the first to challenge the veracity of the earlier models (developed as Arrhenius-
type function) when they used a computer to run simulations on five of the most recognized previous models (including Henriques (32)) and compared the results. They observed that for a given set of burn injury conditions there was significant inter-model variability in the results for the predicted extent of injury and questioned the models validity when extrapolated outside the limits of the experimental data from which they were derived. They assert that as damage functions are not linear it is difficult to extrapolate these models for broad temperature ranges, arguing that as burn temperatures increased, the disparity between models was enhanced. Additionally, both Diller et al. (75) and Orgill et al. (108) argue that Moritz and Henriques model is limited as it does not consider the continued tissue damage that occurs after the initial burn injury when the heat source is removed and the tissue is cooling-down. They cite inconsistencies between the model and experimental data (32) as most apparent for shorter duration burns, which is clinically important as the majority of accidental scalds seen in children are of short duration.

Although Diller’s finite-element models for contact burns in blood-perfused skin (74, 107, 110) improved upon earlier studies, they were not constructed in conjunction with, or verified by, their own experimental data. Addressing this limitation, in 1998 Orgill et al. developed a computer model verified with histological assessments from an accompanying porcine contact burn model (108). They reported a good correlation between histology and the model for burn depth at 24–48 hours post-burn, indicating that burn depth can be predicted by a mathematical model. However, their histologic criteria for depth of tissue injury, ‘depth of gelatinized tissue’, provides only one coarse tissue injury indicator. Also, as only one temperature condition (100ºC) for varying durations of exposure was examined and it was a metal contact burn, extrapolation of these results to more moderate temperature scald burns should be viewed with caution. An important limitation inherent to all mathematical model simulations is that the validity of the results obtained relies entirely on the veracity of the original data used to derive the model. Whilst the sophistication of computer modelling has dramatically advanced, there is still a lack of good quality experimental data for scald burns for a broad range of burn conditions from which these models should be derived.

In more recent years, Diller and his co-workers have published more sophisticated computational models for estimating the time and temperature relationship for different severity scald burns (78, 109). The most up to date and relevant of these is a model which investigates the threshold injury causation curve for severe scald burns (DDPT) (78). The authors describe using ‘standard engineering analysis methods in combination with
Henriques and Moritz experimental and modelling data’ to develop the model. Importantly, this model allows for simulations to be run considering post-burn scenarios such as first-aid treatment. However, the veracity of the results from the model was not verified with their own experimental data. Instead previous burn injury calculations from the literature were used from 3 main sources: Moritz and Henriques (validity of histologic results questionable for dermal injury); Singer and his co-workers (contact rather than scald model); and Diller (the group’s own previous computer model, not experimental). The credibility of such models would be strengthened by good quality experimental data for scald burns.

In order for clinicians to use a mathematical model as a tool to predict burn depth it must be reliable and easy to use. There is currently a lack of such simplified models for use in a hospital setting, where a full simulation is not feasible. In 2011, Abraham et al. (64) extended upon their previous work (104, 109) to develop a simplified algebraic equation to predict burn depth for scald burns (Figure 1-5). They reported a good match ($R^2 = 0.967$) between predicted results from their more complex computer model and their simplified equation. As this mathematical model was entirely based on their previously discussed computational models, the same limitations apply. However, this study provides a good illustration of the usefulness of scald injury prediction data for a clinical setting and in future developing a more user friendly approach such as mobile application software should be considered.

Burn Depth (mm) = $C_0 + C_1T + C_2t + C_3Tt$

$T$= exposure temperature ($^\circ$C), $t$= time (sec), $C$= coefficients

**Figure 1-5 Example of numerical model to predict burn depth**, described by Abraham et al. (64).

1.3.7 Early and accurate assessment of burn depth/severity is important

It is well documented and accepted that burn depth assessment is critical in a clinical setting for determining treatment decisions (7, 8, 105, 111, 112). Early authors such as Entin and Baxter (36), Gursu (111) and Kahn et al.(112) highlight the importance of biopsy at early time points as being a good clinical tool. In their review on diagnosing burn depth in 1992, Heimbach et al. (105) succinctly summarises how advances in treatment options, such as surgical intervention, have magnified the importance of early, accurate burn depth assessment. Heimbach et al. contend that as burns clinicians now have the option for ‘early’
surgical intervention, accuracy in burn depth has become even more critical, whereas previously early surgical options were limited and thus judging the exact depth at early time points was of less consequence. Extending this idea forward to the current day, it is often cited that the ‘challenge’ now is to determine as early and accurately as possible those ‘partial thickness’ burns that will heal spontaneously within 2–3 weeks without scarring and those that will require longer than 3 weeks to heal and are more likely to form hypertrophic scarring (3, 7, 8, 26, 62, 105, 113, 114). Burn wounds that are not predicted to heal within 3 weeks are most commonly treated by early excision and grafting (3). Thus, an important consideration for future studies investigating the time and temperature threshold curve for these clinically relevant deep dermal to full thickness burns, should be to evaluate not only the depth of injury sustained but also the time taken for these burns to heal.

In a research setting, accurate measurement of burn depth is essential. In order to compare different treatment groups it is critical to ensure that the depth of burn for comparison is the same (7). In clinical trials investigating treatment modalities or new diagnostic techniques, burn depth assessment may form the basis for comparisons, thus the ability to accurately measure burn depth will directly affect both the validity and reliability of results obtained (7, 21, 115).

1.3.8 Methods used to assess burn depth and wound healing potential

A full review of the literature regarding assessment of burn injury severity and burn wound healing potential in a clinical setting is beyond the scope of this review and is well summarised in reviews by Devgan et al. (4), Monstrey et al. (7) and Jaskille et al. (8). However, in the context of this thesis, it is important to consider key issues surrounding tissue injury assessment in greater detail for clinical evaluation, biopsy and histology, measurement of tissue perfusion, and measuring wound healing outcomes.

1.3.8.1 Clinical evaluation of burn severity

In clinical practice, the ultimate goal of assessing the extent of tissue injury in the early post-burn period is to identify whether a burn wound is likely to take longer than 2–3 weeks to heal (re-epithelialise) and therefore have a greater chance of developing hypertrophic scarring. Subjective evaluation of the appearance of a burn wound by a clinician is still the most widely used and cost-effective method for assessing burn injury severity (4, 7, 105). However, as noted by many (105, 116, 117), the reliability and accuracy of clinical judgements for depth assessment are sub-optimal. While reliability may vary depending on
the experience of the clinician, it is frequently cited that clinical depth assessment is inaccurate one third of the time (116, 118) and that an overestimation of depth is more common (117). Despite these limitations, clinical evaluation of the burn wound appearance remains the principal method for assessment of tissue injury severity and wound healing potential, whether at the bedside or providing remote expert consultation using digital photographic images. However, in light of concerns regarding precision, specificity and sensitivity; reliance on clinical evaluation alone in a major burn centre setting or for research is questionable.

1.3.8.2 Measurement of tissue perfusion

For the reasons explained above, substantial research effort has been placed on the development of accurate and objective techniques for burn depth assessment. Of the many different methods investigated, measurement of tissue perfusion has been extensively evaluated given the established relationship between depth of burn and microvascular blood flow (119, 120). During the inflammatory phase in the early post-burn period, compared to uninjured skin, for SPT burns there is an increase in blood flow due to local vasodilation. However, for DDPT and FT burns, where the dermal microvasculature is significantly damaged, this increased perfusion does not occur and blood flow is reduced (121, 122). Several techniques to measure tissue perfusion have been studied including: thermography (123, 124), vital dyes (125), Indocyanine green video angiography (126-128) and Laser Doppler-based techniques (117, 118, 121, 129, 130). Over recent years the technique which has gained the widest clinical acceptance is Laser Doppler Imaging (LDI). While use of LDI for assessing tissue perfusion in a clinical setting has several limitations (e.g. patient movement, limited penetration of the laser light due to non-debrided tissue and blisters) (131), it is arguably currently the best available choice as judged by evidence-based estimates of accuracy (4, 115, 132-134) and the fact that LDI is the only diagnostic modality approved by regulatory bodies (including the US Food and Drug Administration) for burn assessment (7).

Cutaneous blood flow can be non-invasively measured by LDI, expressed as arbitrary perfusion units (PU) and displayed as a two-dimensional colour-coded image (six colour palette) (131, 135). An important, but often overlooked distinction noted by others (135, 136), is that LDI does not actually measure burn ‘depth’, rather it represents the degree of blood flow to the wound. This distinction is important, while the correlation between healing potential and LDI has been studied extensively (131, 135, 137), the association between
blood flow and burn depth is less clear. The prospective study conducted in adults and children by Monstrey et al. (131) which validated the use of LDI for predicting burn wound healing potential, found a level of accuracy greater than 90%. Similar levels for LDI accuracy have been reported by others (130, 132) and found to be significantly higher than clinical accuracy. While use of LDI is gaining popularity and is seen by many as an important adjunct to clinical assessment alone, caution is recommended when LDI is used as a standalone measure of burn ‘depth’ (as opposed to healing potential) for research purposes as the exact correlation between blood flow within the burn and burn depth remains unclear.

Another important consideration to be aware of when interpreting LDI images is that, similar to a biopsy, a single LDI image provides only a ‘snap-shot’ of dermal blood flow at a given time point. Contention surrounding the ideal time to perform LDI assessments and the frequency of scanning remains (134). Most of the LDI studies reported do not recommend performing scans earlier than 48 hours post-burn (117, 130, 135) as substantial evolution of the burn injury may occur within the first 48–72 hours post-burn (23). Additionally, Stetinsky et al. (137) contends that in the first 48 hours post-burn, compression of the vascular supply with local oedema may falsely indicate destruction of microvasculature. However Jeng et al. (138), who performed repeat daily LDI scans on 23 patients, found no significant difference in median PU between scans taken on day 1 and subsequent scans. In a more extensive study involving 40 patients with intermediate depth burns, Hoeksema et al. (132) compared the accuracy of LDI assessments on days 0, 1, 3, 5, and 8 and demonstrated that the accuracy of LDI scans continued to improve from day 3 to day 5 post-burn. Stetinsky et al. proposes sequential measurements of the burn injury as a method to improve prediction of healing potential. The authors observed that burns which ultimately healed by 3 weeks showed increasing blood perfusion at each subsequent LDI scan, however, for the group of unhealed burns, perfusion levels were consistently low (137). It is therefore of interest to improve understanding of how changes in perfusion in the burn over time relate to burn depth and healing outcomes.

1.3.8.3 Histological assessment of burn depth and tissue damage

1.3.8.3.1 Overview of reference standards and limitations

Histological assessment of burn wound biopsies has long been considered the ‘gold standard’ for precise burn depth evaluation in both the clinical and research setting (7, 8, 55, 59, 61, 62, 105, 139). However, despite this assertion, there is still no clear consensus (55, 59, 61) regarding the features of histology used as a standard for burn depth
determination. As noted by others in the burns research area (55, 61, 62, 140), a widely accepted international reference standard for histopathological assessment of burns remains a necessity.

Several limitations of biopsy evaluation have been identified: biopsies are a ‘snap shot’ in time of the depth of a wound and don’t account for the dynamic changes in the acute post-burn period (140); they may be prone to sampling error when a non-representative portion of a burn is sampled (4, 7) (i.e. the individual burn may not be uniform in depth); it can be expensive, time consuming (processing/staining and evaluation) and invasive (leaves a scar) (62, 141); tissue shrinkage may occur when the specimen is processed (4, 141); and lastly several authors cite the necessity for an experienced pathologist to analyse the sample as a limitation (4, 7, 62, 141) which is further compounded by the subjective nature of the assessment. As Watts et al. (140) suggests, histopathological measurement of burn depth can also be an inexact science, which can be subjective and open for interpretation. Therefore, before proceeding to further examine the relationship between burn temperatures, duration of exposure and tissue injury severity, it will be necessary to review issues surrounding the histopathological markers used to assess tissue damage.

1.3.8.3.2 Issues surrounding the histopathological markers used to assess burn depth and tissue damage

1.3.8.3.2.1 Depth of injury varies depending which dermal elements are assessed

There is consensus in the literature to support observations that the extent of injury varies between different dermal elements (55, 62, 140, 142) and that accurate measurement of burn depth should include evaluation of several dermal elements e.g. endothelial cells, collagen, hair follicles and glands. Singer’s group (55) conducted the most comprehensive research in this area and propose that for each dermal element there are two factors that may be involved in determining the depth of injury; heat conductivity and thermal sensitivity. The dermal components commonly cited as markers for depth of thermal injury are collagen alteration (7, 46, 105, 143), vascular patency (1, 23, 55, 112, 140), and follicular cell damage (1, 23, 144). To a lesser extent mesenchymal cells and smooth muscle have also been reported as injury markers (55, 142). However, contention exists regarding not only the optimum time points post-burn for biopsy and the most effective staining techniques for identifying cell injury to different dermal elements (59, 61, 145), but also the relative importance of specific dermal elements as markers for assessing the overall severity of a burn (55, 142).
The reliability of collagen alteration as a diagnostic marker for burn depth, particularly at early time points for low to moderate temperature burns, has been questioned (61, 140). This is important in the context of this review as in contrast to most contact burns, many scald burns are sustained via moderate temperatures (60–80°C). Pioneering studies performed by Chvapil et al. (143) in the 1980s, which were conducted in pigs using a brass block heated to temperatures in excess of 200°C, established that the depth of changes to collagen fibre density (leading to abnormal staining using a Massons’ trichrome stain) was proportional to the time and temperature of exposure. Since that time other investigators, using similarly high contact burn temperatures, also reported observing a clear line of demarcation between denatured and uninjured collagen using special stains (37, 46, 146). As discussed earlier in this review, only one study has directly compared the histopathology of scald compared to contact burns in a porcine model. Brans et al. (37) observed that whilst a clear line of demarcation could be seen with a contact burn (170°C), scald burns (for the same duration of exposure) showed a more intermingled pattern of collagen damage. More recent and comprehensive investigations by Hirth et al. (61) comparing the histopathologic staining of low temperature burns in a porcine contact burn model support the idea that collagen injury may be an insensitive marker for determining injury depth in low temperature scald burns. Hirth’s group asserts that higher temperatures may be required to irreversibly denature collagen. For example, with contact burns made at 80°C for 20 seconds they observed deep partial thickness to full thickness injuries when defined by endothelial and epithelial necrosis, and vascular occlusion. However, when evaluated for collagen denaturation (observed by colour change using Massons’ trichrome (MT), Elastin Von Gieson (EVG) and Haematoxylin Phloxine Saffron (HPS) stains), the burns were identified as more superficial partial thickness. Generally, cells will start to denature between 44°C (147) and 47°C (128), however, collagen is inherently more stable to thermal insult and may not begin to denature until temperatures in excess of 60°C (148) to 65°C (105). Therefore, when developing methodologies for accurate determination of burn depth in low temperature burns, caution should be given to interpreting collagen damage in the reticular dermis as a marker of injury depth.

The importance of assessing vascular patency as a marker for burn depth was first proposed by Moritz (33), supported by Jackson (23) and Kahn et al. (112) and is discussed more recently by Singer et al. (55, 57) and Watts et al. (140). Singer et al. (57) proposed placing emphasis on measuring the depth of endothelial cell necrosis as a critical factor, reporting
that endothelial injury was the easiest and most consistent measure of depth of injury to
distinguish between various burn injury conditions. Singer et al. also found a strong
correlation between endothelial cell injury at 1 hour post-burn and ultimate scar surface area
at 28 days (57), proposing dermal ischemia as a key mechanism of burn wound progression.
More recently Hirth et al. (149), a collaborator with Singer, further validated this theory by
demonstrating that endothelial cell necrosis in the zone of stasis is a strong predictive
marker for final histologic level of injury at 7 days post-burn. For porcine scald burn models
there is a paucity of literature investigating the relative importance of vascular patency as a
marker of burn depth. Interestingly, Brans et al. (37) observed in their scald model that for
some intermediate depth burns the superficial vasculature could still be intact even though
the deep dermal plexus was damaged. Given the current emphasis placed on developing
clinical modalities which measure tissue perfusion for burn injury depth diagnosis (e.g. Laser
Doppler Imaging), further investigations in this area for scalds are warranted.

1.3.8.3.2.2 The timing post-burn of histological assessment will affect results
Ideally, early (1–4 hours post-burn) histological analysis of a burn wound would provide an
accurate definition of irreversible depth of tissue injury. However, as discussed by Devagan
et.al (4) and Monstrey et al. (7) in reviews of burn depth assessment modalities, in reality
such an assessment may be too simplistic. Both reviews contend the key challenge for early
post-burn histological analysis is to differentiate between structural and functional cell
damage given that some cells which appear structurally viable may in fact be non-functional.
The recent work of Hirth et.al (61, 149) confirms the importance of careful interpretation of
histologic appearance in the early post-burn period, claiming that histologic appearance of
cell death can be delayed (by hours or even days) and proposes the use of staining methods
which can rapidly identify markers of injury that are followed by loss of cell viability. Due to
the dynamic and progressive nature of burn wounds, the ability of microscopic analysis to
distinguish between a cell which is injured/damaged but may or may not proceed to die is a
critical area of investigation. This area of research is relevant to both clinicians and
researchers alike and is currently inadequately addressed.

Since Jackson’s (23) pivotal work in the 1950s proposing a ‘zone of stasis’ which initially
contains patent sub-papillary vessels but progresses within the first 24 hours post-burn to
complete stasis of the superficial capillaries, the theory of burn wound progression has
gained widespread acceptance. In the 1990s Nanney et al. (145) suggested that the
progression of the burn zone tissue damage extends beyond the first 24 hours post-burn.
Using an objective method, Vimentin staining (immunomarker) and a traditional staining method (Trichrome) Nanney’s group demonstrated that compared to day 1, wounds from post-burn day 3 and 4 were significantly deeper. In contrast, a study by Lanier et al. (150) showed (using data from their porcine hot comb burn model) that irreversible changes at the cellular level may in fact occur much earlier post-burn, reporting significant progression of vascular endothelial cell necrosis between 1 and 4 hours post-burn. Hirth et al. (61) supports Lanier’s theory that burn injury progression may occur rapidly (within the first 24 hours), suggesting that Vimentin staining may underestimate burn depth at early post-burn time points (1–24 hours) and argues that interpretation of burn depth at earlier time points by Nanney et al. may not have been valid. Whilst the theory of burn wound progression is now widely accepted, an exact description of the timing and mechanisms behind the cellular and molecular events responsible remains elusive. This information is essential for the development of early intervention therapies designed to limit burn injury progression.

1.3.8.3.3 Different histological staining techniques are used to identify cell and tissue damage

Even with advances in Immunohistochemical (IHC) staining methods over the last 30 years (141), the majority of investigators routinely utilise histochemical stains performed on biopsy samples which have been formalin-fixed and embedded in paraffin prior to staining. A comprehensive review of all stains used for histopathological analysis and imaging of burns is beyond the scope of this review and is well summarised in a recent study by Hirth et al. (61). In their study, the authors compared adjacent sections from burned porcine skin (using both frozen and formalin-fixed sections) to examine the strengths and weaknesses of commonly used histochemical stains for burn depth analysis. For overall assessment, they recommend the general utility of standard Haematoxylin and Eosin (H & E) and Haematoxylin Phloxine Saffron (HPS) for identifying cell and tissue damage in burned tissue.

One of the disadvantages of routine histochemical stains is their inability to differentiate between cells which may appear morphologically intact but are actually injured to the extent that they are metabolically inactive i.e. cell viability (151-153). An alternative method proposed to overcome this limitation is the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method of staining fresh biopsy samples (152). This method for live tissue staining has the potential to rapidly assess cell viability, however, there is a paucity of studies regarding live tissue MTT staining for burn depth assessment. A more
detailed review of work in this area is discussed in Appendix A of this thesis.

1.3.9 Assessment of wound healing

The importance of time to healing as an outcome measure for burn injury severity has been discussed in the preceding sections of this review. While precise quantification of depth of tissue injury is desirable for experimental models of thermal injury, arguably, translation of results is strengthened by using the more clinically relevant outcome of time to healing as a measure of burn injury severity. In the burns research area both macroscopic and microscopic assessments for wound re-epithelialisation are used to evaluate wound healing. However, both of these methods have limitations which should be considered and are discussed below. The reliability and validity of assessment for re-epithelialisation is extremely important for studies where time to healing is the primary (and often only) outcome measured.

1.3.9.1 Clinical assessment of wound healing

Gross wound healing is evaluated by visually assessing the wound and recording the rate of re-epithelialisation either subjectively (judgement by experienced observer) or objectively (using wound measurement techniques). Typically wounds are considered re-epithelialised, ‘healed’, in the presence of a dry, non-glossy, opalescent covering (154, 155) involving ≥ 95% of the wound area (2). Of the objective measurement techniques, digital planimetry using a wound measurement system called Visitrak™, has been used most extensively and validated in both human (156) and animal (2, 157) studies. More recently the use of 3D photography (stereophotogrammetry) has been explored as a non-invasive technique for measuring burn wound area (156). However, it should be understood that these objective methods only quantify assessments made from visual inspection of the wound and an experienced observer is still required to determine what areas to measure. In human studies, the experience of the observer has been shown to impact the reliability of visual wound assessments (158). This suggests that for research purposes, reliance on gross assessment alone is less than optimal and where feasible, such as in animal models, evaluation of re-epithelialisation microscopically should also be considered.

1.3.9.2 Microscopic assessment of wound healing

Microscopic assessment of wound healing involves examination of stained tissue sections to determine the proportion of the section which has re-epithelialised (observed as
neoepidermis). Although microscopic evaluation of re-epithelialisation by means of a skin biopsy is considered the gold standard and is known to have high inter-observer reliability (155), there are few studies comparing clinical and histological assessments. In a porcine model of burn wound healing, Singer et al. (155) found a poor agreement between gross clinical assessment and microscopically determined wound re-epithelialisation. When measuring the re-epithelialisation of a 1cm diameter circle in the centre of the burn, the authors observed for some wounds which a clinician considered completely re-epithelialised there was in fact no evidence of neoepidermis on microscopic analysis. While in the study by Singer et al. the area to be biopsied was standardised, this is not always the case and a limitation of biopsies often cited anecdotally by clinicians is the sampled area may not be representative of the wound as a whole. Additionally, where the rate of re-epithelialisation is to be recorded over time, repeat biopsies may potentially disrupt wound healing affecting interpretation of results. Therefore, while researchers should avoid reliance on gross macroscopic assessment alone for measuring re-epithelialisation, additional measures such as biopsy should be timed with care and interpreted with caution.

1.3.10 Conclusion

The literature clearly demonstrates that even with major advances in burns diagnostics, treatments and prevention over the last 70 years, thermal injuries remain a significant global health concern. While the results of epidemiological studies from burn centres both in developed and developing countries continue to confirm the importance of scalds as a mechanism of thermal injury, particularly in children, current burns research is heavily skewed towards using contact burn models. Some porcine contact burn model studies discussed in this review by Singer et al. (44, 55, 57) and Hirth et al. (61) substantially increase understanding of the relationship between burn temperature, duration of exposure and tissue injury severity. However, there remains a deficiency of comprehensive studies using scald as a mechanism of injury, particularly for porcine models.

One could speculate that the reason there has been so little work done in this area in recent years may be that for too long the seminal work of Moritz and Henriques (1), performed in the 1940s, has been accepted as definitive. Whilst their work was certainly noteworthy for its time, the validity of their results for describing the time and temperature threshold for deep dermal injuries must now be questioned. With advances in treatment options available for burns, such as surgery and grafting, the importance of accurate and early diagnosis of
burn depth is even more critical. In this context, with the focus on differentiating between SPT and DDPT burns, the histological criteria used by Moritz and Henriques to categorise extent of thermal injury (trans-epidermal necrosis) is outdated. To date no other investigators have comprehensively re-examined this work using current histologic techniques in a scald model. Despite their limitations, the results obtained from Moritz and Henriques original investigations (1, 32, 33) are still quoted in the most current burn prevention articles and researchers developing mathematical burn models are still relying on this data.

While no experimental animal model is a perfect representation of humans, the literature establishes that an *in vivo* porcine burn model is the model of choice for thermal investigations of this nature. However, there remains contention over the reliability of the various types of porcine models and their limitations. As mentioned, there are very few porcine scald models. Apart from Moritz and Henriques scald model, none of these porcine scald models can apply water maintained at a consistent temperature for long durations. Additionally, there is virtually no experimental, evidence-based data regarding how an increase or decrease in the skin thickness (with age, and/or anatomical location) may affect the susceptibility of the skin to thermal insult. Further knowledge in these areas is essential globally for guiding any recommendations regarding the setting of standards for hot water safety.

The literature surrounding histological assessment of thermal injury is vast. Many of the staining methods used are well described across several studies, in particular the recent work of Hirth *et al.*(61) and Singer *et al.*(159). However, there is currently a substantial gap in our understanding of how these different histochemical and IHC techniques may perform in a porcine scald burn model. In addition, conflicting opinions exist concerning not only the reliability of stains for demonstrating thermal damage to different dermal elements but also for different times post-burn. Consideration of these discrepancies is essential for optimising the experimental design of future studies. Fresh tissue staining (as opposed to fixed) is used extensively in myocardial and cerebral ischemia models, however there is no literature available which examines its use for burn depth assessment in moderate temperature thermal injuries (such as scalds) even though this would be advantageous.

This review demonstrates that while histological assessment of tissue injury plays a key role in providing valid evidence-based scald burn injury prediction data, for translation of results to humans it is also essential to relate findings to the more clinically relevant outcome of
time to healing. While biopsies are standard to animal studies, they are rarely performed in a clinical setting to aid treatment decisions. Conversely, non-invasive diagnostic modalities for burn severity assessment have gained in popularity, namely, measurement of blood flow to the burn area. However, how changes in perfusion of the burn wound in the early post-burn period (0–7 days) relate to burn depth (determined histologically) and ultimate healing outcome remains poorly understood.

In summary, the key issue identified in this review is that despite the importance of scald burns globally, the most basic data involving the relationship between burn temperature, duration of exposure and tissue injury severity for scald injury have not been validated by comprehensive histological analysis and time to healing investigations. To address this, the development of a reliable porcine scald model with the ability to test for a broad range of burn conditions (different temperatures and durations of exposure) is required. Through using this model, several other key gaps in our knowledge regarding scald burns may be investigated such as: the pathophysiology of burn wound progression; the effect of skin thickness on thermal susceptibility; and the reliability of different histochemical, IHC and fresh tissue staining techniques for burn depth assessment. This area of research is essential to guide global burn prevention strategies and to improve the overall management and treatment of scald injuries.

1.4 Thesis Aims

The purpose of this thesis was to provide updated, evidence-based scald injury prediction data by validating the relationship between water temperature, duration of exposure and tissue injury severity. Specifically, this research project aimed to establish the burn conditions which cause clinically relevant severe paediatric scald injuries. This was achieved through conducting experimental animal studies using a porcine thermal injury model. The similarities between pig skin and human skin are well described (1, 42, 67, 69), making this an ideal model for translation of results to humans.

Firstly, it was important to ascertain what burn conditions (burn temperature and duration of exposure) have been described by previous porcine models to create different severity burns. Many different models exist, nevertheless, it was unknown whether a pooled analysis of these studies could provide acceptable estimates for the burn conditions to cause severe scald injuries.
Secondly, to test burn conditions identified by the literature and clinical relevancy, development of a consistent and reliable porcine scald model was required. Using this model, the purpose of the experimental animal studies was to determine the burn conditions required to sustain a severe spill/splash or immersion scald injury. On the basis of these studies, a key objective of this research project was to develop clear burn injury prediction estimates to guide scald burn prevention strategies, advise regulators setting product safety standards, and also to inform medicolegal judgements where an inflicted mechanism injury is suspected.

Thirdly, it was also important to improve understanding of heat conduction in skin and consider how this relates to tissue damage and burn injury severity. Using the quality experimental data provided by this study, this research project aimed to inform development of mathematical modelling to investigate how the thermal properties of skin depend on burn duration, burn temperature, and skin thickness.

1.5 Thesis Overview

During my PhD candidature, priority was given to publishing as soon as the work was completed, therefore this thesis contains work which has already been peer-reviewed and published in several different journals. This thesis is presented as eight chapters, with chapters 2, 3, 4, 5 and 6 based on published manuscripts.

Chapter 1 consists of an introductory section providing a general overview of paediatric burn injuries and a comprehensive literature review examining current issues associated with burn injury severity evaluation and with predicting the healing potential of burn wounds. Chapter 2 is a systematic review of porcine burn models (published in the International Wound Journal) which details the strengths and weaknesses of existing experimental evidence regarding the burn conditions reported to cause burn injuries of different severity.

The main methods and results for this thesis are presented as experimental chapters 3 to 7 and Appendix A:

Chapter 3 - describes in detail the methodological approach taken to develop a novel porcine scald model (published in the journal PLoS One).

Chapter 4 - examines the thermal properties of skin using a mathematical model to interpret temperature profile data from the experimental animal studies (published in the International Wound Journal).

Chapter 5 - details how discrepancies between in vivo and ex vivo skin thickness measurement techniques were evaluated (published in the journal Wound Repair and Regeneration).

Chapter 6 - describes the main results and findings from the experimental animal studies. Scientific evidence describing the burn conditions for clinically relevant deep dermal scald injuries is provided and updated scald injury prediction data is presented (published in the journal Wound Repair and Regeneration).

Chapter 7 - presents evidence from clinical observations for the burn conditions and circumstances for severe scald injuries in children, a retrospective chart and database review of Queensland’s major paediatric burn treatment centre is described.

Appendix A - describes and discusses some of the additional histopathological staining methods evaluated for this research project.

Each experimental chapter of this thesis has its own discussion. Therefore, the final chapter of this thesis, chapter 8, provides an independent overview of all the work and includes: a synthesis of the main findings, discussion regarding translatability of results to clinical practice and recommendations, study limitations, and directions for future research.
Chapter 2    Systematic review of porcine thermal injury models

This chapter is based on a published paper in the International Wound Journal.

Citation:

2.1 Chapter foreword

As described in the preceding chapter, there are numerous porcine burn models reported in the literature. Before embarking on development of a new model for this research, it was important to consider whether a systematic review of existing porcine burn models could provide information regarding the burn conditions reported to cause burns of different severities. Additionally, as the literature review indicated, the effect of mechanism of injury on burn severity is poorly understood, therefore it was of particular interest to evaluate how porcine models using different methods to create thermal injuries compare.

This chapter is based on the publication titled ‘Comparing the reported burn conditions for different severity burns in porcine models. A systematic review’ and details one of the methodological approaches taken in this thesis to review the current field of burn injury prediction and obtain evidence for the burn conditions required to cause a severe deep scald injury using an appropriate animal model. This encompasses a systematic review of 42 porcine burn studies describing depth of burn injury with histological evaluation. The study in this chapter also assessed the methodological and outcome measurement limitations of existing experimental porcine burn model studies which aided the design and optimisation of the novel porcine scald burn model presented in chapter 3.
Comparing the reported burn conditions for different severity burns in porcine models. A systematic review.

2.2 Abstract

There are many porcine burn models which create burns using different materials (e.g. metal and water) and different burn conditions (e.g. temperature and duration of exposure). This review aims to determine whether a pooled analysis of these studies can provide insight into the burn materials and conditions required to create burns of a specific severity. A systematic review of 42 porcine burn studies describing depth of burn injury with histological evaluation is presented. Inclusion criteria included thermal burns, burns created with a novel method or material, histological evaluation within 7 days post-burn and method for depth of injury assessment specified. Conditions causing deep dermal scald burns compared to contact burns of equivalent severity were disparate, with lower temperatures and shorter durations reported for scald burns (83°C for 14 seconds) compared to contact burns (111°C for 23 seconds). A valuable archive of the different mechanisms and materials used for porcine burn models is presented to aid design and optimisation of future models. Significantly, this review demonstrates the effect of mechanism of injury on burn severity, and that caution is recommended when burn conditions established by porcine contact burn models are used by regulators to guide scald burn prevention strategies.

2.3 Introduction

Burns due to accidental or non-accidental exposure to a thermal insult are common and occur via multiple mechanisms including flame, contact, scald and radiant heat exposure. Consequently, over the last century, a considerable amount of research has been conducted to investigate the burn conditions (temperature and duration of exposure) required to sustain different severity burns. Animal thermal injury models are used extensively to study burn wounds (65, 160) and provide important in vivo data, replicating the complex pathophysiology that occurs in living skin after a thermal insult. Porcine burn models have been extensively validated and are widely acknowledged to be the animal model of choice for thermal injury investigations (1, 65, 69, 70) given the anatomical and physiological similarities between pig skin and human skin (44, 67, 69, 70).

A major concern regarding the use of porcine burn models is the challenge of creating...
reproducible burns of the same depth and severity. Numerous investigators have published porcine burn models, describing the burn conditions required to create a desired depth or severity of injury (2, 39, 43, 44, 46, 54, 83, 161). The various strengths and weaknesses of the different techniques and reproducibility of burn creation has been described previously (43, 65, 162). However, there is limited discussion regarding the substantial discrepancies between studies for the burn conditions reported to create equivalent depth burns. For example, in studies researching burn wound healing and hypertrophic scar formation, a porcine model of deep dermal partial thickness (DDPT) burns is highly desirable and multiple models are reported (2, 43, 46, 57, 163). In these studies the burn conditions reported for equivalent DDPT burns varies widely from 190°C for 21 seconds (163) to 92°C for 15 seconds (2). Therefore, it is of specific interest to understand whether a pooled analysis of studies could allow for conclusions to be made regarding the burn conditions required to sustain a clinically relevant DDPT burn. Additionally, knowledge regarding the strength of this effect could be used to power future studies.

Experimental studies comparing different mechanisms of thermal injury are scarce. Brans et al. compared tissue damage caused by contact burns and scald burns (37) in a porcine model and provided histological evidence suggesting their pathophysiology differs. It is important to reflect on whether novel therapies, interventions and diagnostic modalities examined in burn models using contact with metals as the mechanism of thermal injury can be more broadly applied to burns sustained via different mechanisms such as scald and flame injury. Additionally, results for burn conditions established by porcine models are often used by regulators to guide burn prevention strategies. However, the validity of using results from a contact burn model to inform scald and/or flame burn prevention policy is unknown.

The primary aim of this review was to provide both a comprehensive qualitative and quantitative appraisal of the literature regarding porcine burns created via different mechanisms with different materials. The secondary aim was to compare the burn conditions reported to create wounds with specified burn depths [superficial, superficial partial thickness (SPT), DDPT and full thickness (FT)] determined histologically, with particular emphasis given to DDPT burns. To our knowledge, this is the first time a systematic review of porcine burn models has been conducted, evaluating whether a pooled analysis for the specific outcome measure, histologic depth of damage, can be performed. Additionally, this review is one of only a few systemic reviews of experimental animal studies where the methodological quality of the included studies is quantitatively evaluated using a
modification of the ARRIVE guidelines (164).

2.4 Methods

A systematic review of the published literature on porcine burn models was conducted. Inclusion criteria were experimental, in vivo, porcine, skin burn models and published in English. Full inclusion and exclusion criteria are listed in Table 2-1.

Table 2-1 Inclusion and exclusion criteria for eligible studies

<table>
<thead>
<tr>
<th>Inclusion criteria</th>
<th>Exclusion criteria</th>
</tr>
</thead>
<tbody>
<tr>
<td>• Porcine only</td>
<td>• Ex vivo</td>
</tr>
<tr>
<td>• In vivo</td>
<td>• Lung, gastrointestinal or systemic burn models</td>
</tr>
<tr>
<td>• Cutaneous or skin burn model</td>
<td>• Chemical burns, laser burns, radiation burns, cold burns</td>
</tr>
<tr>
<td>• Assessment of depth of tissue injury specified</td>
<td>• Studies quoting use of a previously described burn infliction method</td>
</tr>
<tr>
<td>• Histologic evaluation for depth of burn injury for at least 1 time point (within 7 days) post-burn</td>
<td>• Full-text unavailable in English</td>
</tr>
<tr>
<td>• Thermal burns expressed as temperature</td>
<td></td>
</tr>
<tr>
<td>• Novel method or material for burn infliction and/or if method is based on a previous model (quoted) but material is significantly altered or modified</td>
<td></td>
</tr>
<tr>
<td>• English</td>
<td></td>
</tr>
</tbody>
</table>

2.4.1 Data sources and article selection

The databases searched included EMBASE, PUBMED and Web of Science from the earliest time until August 2016. The detailed search strategy for each database is given in Table 2-1. Search terms included; ‘pigs OR pig OR swine OR swines OR piglets OR piglet’ AND ‘animal OR model OR experimental study OR in vivo’ AND ‘burns OR burned OR thermal injury OR burn OR thermal studies’.

The search was supplemented by studies identified by cross referencing and key author searches. Studies for inclusion/exclusion were assessed by two reviewers (CJA and LC) independently. After removal of duplicates, articles were screened for inclusion based on title and abstract. Full-text versions were then obtained for the remaining articles and assessed for eligibility by both reviewers. Only articles where full-text was available in English were included in the review. Other than reviews, all study types including doctoral
thesis and congress abstracts were included, if full-text was available.

Table 2-2 Database search strategy

<table>
<thead>
<tr>
<th>Database</th>
<th>Search terms</th>
</tr>
</thead>
</table>
                | 2. (Disease Models, Animal [MeSH]) OR (Model[tiab] OR experimental study[tiab] OR “in vivo”)  
                | 4. #1 and #2 and #3                                                        |
| EMBASE         | 1. Pigs:ti,ab OR pig:ti,ab OR swine:ti,ab OR swines:ti,ab OR piglet:ti,ab OR ’swine’/exp  
                | 2. ’burn’/exp OR Burns:ti,ab OR burned:ti,ab OR “thermal injury”:ti,ab  
                | 3. ’Animal model’                                                            
                | 4. #1 and #2 and #3                                                        |
| Web of Science | 1. TS=(Pigs OR pig OR swine OR swines OR piglets OR piglet) AND TS=(Animal OR Model OR “experimental study“ OR “in vivo”) AND TS=(Burns OR burned OR “thermal injury” OR burn OR Burn* OR “thermal studies”)  
                | 2. TS=guinea                                                                
                | 3. #1 NOT #2                                                                
                | 4. English only                                                              |

2.4.2 Assessment of quality of studies

Quality was rated using a modified checklist developed from the ARRIVE guidelines for reporting animal research (164) with items 11 and 14 not assessed (as they are more relevant to randomised therapeutic trials and were not deemed applicable here). Items for quality assessment are listed in Table 2-3. Each item in the checklist was given a quality score where 0 = ALL criteria inadequate or not mentioned, 1 = SOME criteria missing or unclear, 2 = ALL criteria adequate and clear. For item 17 adverse events, not applicable (n/a) was recorded if there was no indication an adverse event had occurred.
Table 2-3 List of items for quality assessment of included articles. The ARRIVE guidelines (Kilkenny et al. 2014) were modified with items 11 and 14 not assessed.

<table>
<thead>
<tr>
<th>Item</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Title</td>
<td>1 Provide as accurate and concise a description of the content of the article as possible</td>
</tr>
<tr>
<td>Abstract</td>
<td>2 Provide an accurate summary of the background, research objectives, key methods, principal findings and conclusions of the study</td>
</tr>
<tr>
<td>Background</td>
<td>3 Include sufficient scientific background to understand motivation and context for the study, explain the experimental approach, and relevance to human biology</td>
</tr>
<tr>
<td>Objectives</td>
<td>4 Clearly describe primary and any secondary objectives of the study</td>
</tr>
<tr>
<td>Ethical statement</td>
<td>5 Description of compliance to national regulatory principles and or relevant licenses, that cover the research</td>
</tr>
<tr>
<td>Study design</td>
<td>6 Methods to reduce bias explained e.g. randomisation, blinding, order in which burns were created. Number of animals used explained. Time between burn creation and sampling clearly outlined.</td>
</tr>
<tr>
<td>Experimental procedures</td>
<td>7 Provide precise details of all procedures carried out and description of equipment: How e.g. type of burning device, heat source, temperature, duration Where e.g. site of burns What e.g. number and size of burns</td>
</tr>
<tr>
<td>Experimental animals</td>
<td>8 Provide details of animals used including; species, sex, age, weight</td>
</tr>
<tr>
<td>Housing and husbandry</td>
<td>9 Type of facility, how animals housed and fed. Describe any welfare assessments made and pain relief medications used (for pigs undergoing recoverable surgeries)</td>
</tr>
<tr>
<td>Sample size</td>
<td>10 Specify number of replicates of each burn condition, provide details of any sample size calculation used (e.g. power analysis)</td>
</tr>
<tr>
<td>Allocating animals to experimental groups</td>
<td>11 Allocation animals to experimental groups - randomisation or matching, order in which animals were treated and assessed</td>
</tr>
<tr>
<td>Experimental outcomes</td>
<td>12 Clearly define the primary and secondary experimental outcomes assessed e.g. burn depth or severity</td>
</tr>
<tr>
<td>Statistical methods</td>
<td>13 Provides details of the statistical methods used for each analysis</td>
</tr>
<tr>
<td>Baseline data</td>
<td>14 Characteristics and health status of animals - microbiological status, drug-or test-naive before treatment</td>
</tr>
<tr>
<td>Numbers analysed</td>
<td>15 Absolute numbers in each group included in each analysis, explanation if any animals, burns or data were excluded</td>
</tr>
<tr>
<td>Outcomes and estimation</td>
<td>16 Results for each analysis with a measure of precision</td>
</tr>
<tr>
<td>Adverse events</td>
<td>17 Give details of any adverse events (if relevant)</td>
</tr>
<tr>
<td>Interpretation/scientific implications</td>
<td>18 Interpret results, refer to hypothesis, comment on limitations or sources of bias</td>
</tr>
<tr>
<td>Generalisability/translation</td>
<td>19 Relevance to human biology discussed</td>
</tr>
<tr>
<td>Funding</td>
<td>20 Disclose funding sources and any conflicts of interest</td>
</tr>
</tbody>
</table>
2.4.3 Data extraction and analysis

Data extraction was completed for all included studies by one reviewer (CJA). Data for extraction followed the PICOTS format (participants, intervention, comparison, outcome, time and study design) as outlined in Table 2-4.

Table 2-4 Data collected for analysis from included articles

<table>
<thead>
<tr>
<th>Data collected</th>
<th>Details</th>
</tr>
</thead>
<tbody>
<tr>
<td>Participants</td>
<td>Animals:</td>
</tr>
<tr>
<td></td>
<td>- breed, age, gender, weight, skin thickness</td>
</tr>
<tr>
<td></td>
<td>- number of pigs in study</td>
</tr>
<tr>
<td>Intervention</td>
<td>Burn creation:</td>
</tr>
<tr>
<td></td>
<td>- mechanism of thermal injury</td>
</tr>
<tr>
<td></td>
<td>- location, size and number of burns per pig</td>
</tr>
<tr>
<td></td>
<td>- apparatus used e.g. type of material, heat source, pressure control</td>
</tr>
<tr>
<td></td>
<td>- burn conditions, temperature (°C) and duration of exposure (sec)</td>
</tr>
<tr>
<td>Comparison</td>
<td>Within animal normal unburned skin as control</td>
</tr>
<tr>
<td>Outcome</td>
<td>Burn depth or severity measured histologically:</td>
</tr>
<tr>
<td></td>
<td>- quantitative or qualitative</td>
</tr>
<tr>
<td></td>
<td>- staining method used</td>
</tr>
<tr>
<td>Time</td>
<td>All skin biopsies collected within 7 days of burn creation</td>
</tr>
<tr>
<td>Study Design</td>
<td>All included</td>
</tr>
</tbody>
</table>

To enable comparisons between studies for burn injury severity, burns were classified by currently accepted nomenclature: superficial, SPT, DDPT and FT burns (21, 31). Where alternate classifications were given, such as first degree, second degree and mid-dermal, the closest equivalent burn injury classification was assigned. For studies where burn severity was reported quantitatively in tables or graphs, a value of ≥ 60% relative injury to dermis signified a DDPT burn (165).

Descriptive statistics such as mean ± SD were used to report the characteristics of the animals and wounds. Meta-regression was considered where appropriate. Analysis was
performed using Graphpad Prism V6 (GraphPad Software, Inc, California, USA) and SPSS 24 (IBM Corporation, Armonk, N.Y., USA).

2.4.4 Outcome measures

The primary outcome measure assessed was burn conditions reported to create superficial, SPT, DDPT, FT burns, as determined histologically. Calculations for the average temperature and duration of exposure required to cause different severity burns for the different mechanisms of injury were made from the available data.

2.5 Results

2.5.1 Study Selection

The work flow of studies included in the review is outlined in Figure 2-1. Reasons for exclusion after full text assessment (n = 99) included: method not novel (n = 59), unable to obtain full text article in English (n = 16), not in vivo (n = 3), histology evaluation by day 7 not reported (n = 6), assessment for depth of injury was not adequately specified (n = 9), not a thermal burn expressed as a temperature (n = 4) and reviews (n = 2). A final total of 42 studies were included Table 2-5.
Figure 2-1 Flow diagram for search and selection strategy of included articles.
Table 2-5 The animal demographics for included studies (n = 42). If values are given as a range, the mean for that range is presented.

<table>
<thead>
<tr>
<th>Author (ref)</th>
<th>Year</th>
<th>Study Type</th>
<th>Burn Mechanism</th>
<th>Number of pigs</th>
<th>Breed of pig</th>
<th>Average age of pigs (wks)</th>
<th>Average weight of pigs (kg)</th>
<th>Average skin thickness (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brans et al. (37)</td>
<td>1994</td>
<td>pathophysiology/histology</td>
<td>scald, contact</td>
<td>1</td>
<td>Yorkshire</td>
<td>.</td>
<td>42.8</td>
<td>.</td>
</tr>
<tr>
<td>Branski et al. (83)</td>
<td>2008</td>
<td>model development</td>
<td>contact</td>
<td>3</td>
<td>Yorkshire</td>
<td>52</td>
<td>52</td>
<td>.</td>
</tr>
<tr>
<td>Breuing et al. (166)</td>
<td>1992</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>5</td>
<td>Yorkshire</td>
<td>14</td>
<td>47.5</td>
<td>2.75</td>
</tr>
<tr>
<td>Brink et al. (163)</td>
<td>1986</td>
<td>diagnostic modalities</td>
<td>contact</td>
<td>5</td>
<td>Mini-pig</td>
<td>24</td>
<td>15</td>
<td>2</td>
</tr>
<tr>
<td>Brown et al. (167)</td>
<td>1986</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>.</td>
<td>Yorkshire</td>
<td>.</td>
<td>7.5</td>
<td>.</td>
</tr>
<tr>
<td>Chvapil et al. (143)</td>
<td>1984</td>
<td>pathophysiology/histology</td>
<td>contact</td>
<td>4</td>
<td>Yorkshire</td>
<td>.</td>
<td>12.5</td>
<td>.</td>
</tr>
<tr>
<td>Cuttle et al. (2)</td>
<td>2006</td>
<td>model development</td>
<td>contact</td>
<td>.</td>
<td>Large white</td>
<td>.</td>
<td>17.5</td>
<td>.</td>
</tr>
<tr>
<td>Danilenko et al. (168)</td>
<td>1995</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>.</td>
<td>Mini-pig - Yucatan</td>
<td>.</td>
<td>50</td>
<td>.</td>
</tr>
<tr>
<td>Durham et al. (169)</td>
<td>1993</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>3</td>
<td>.</td>
<td>.</td>
<td>38</td>
<td>.</td>
</tr>
<tr>
<td>Eldad et al. (170)</td>
<td>2003</td>
<td>prevention</td>
<td>flame</td>
<td>9</td>
<td>White</td>
<td>14</td>
<td>21</td>
<td>.</td>
</tr>
<tr>
<td>Eldad et al. (171)</td>
<td>1991</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>6</td>
<td>Large white X Landrace</td>
<td>.</td>
<td>30</td>
<td>.</td>
</tr>
<tr>
<td>Farhangkhoee et al. (59)</td>
<td>2012</td>
<td>pathophysiology/histology</td>
<td>contact</td>
<td>7</td>
<td>Yorkshire</td>
<td>.</td>
<td>37.5</td>
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</tr>
<tr>
<td>Gaines et al. (46)</td>
<td>2013</td>
<td>model development</td>
<td>contact</td>
<td>4</td>
<td>Yorkshire</td>
<td>12</td>
<td>22.5</td>
<td>.</td>
</tr>
<tr>
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<td>contact</td>
<td>11</td>
<td>Yorkshire</td>
<td>.</td>
<td>25</td>
<td>2.5</td>
</tr>
<tr>
<td>Goans et al. (173)</td>
<td>1977</td>
<td>diagnostic modalities</td>
<td>contact</td>
<td>.</td>
<td>Yorkshire</td>
<td>.</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Gurfinkel et al. (80)</td>
<td>2010</td>
<td>model development</td>
<td>radiant heat</td>
<td>7</td>
<td>Large white</td>
<td>.</td>
<td>35</td>
<td>.</td>
</tr>
<tr>
<td>Gursu et al. (111)</td>
<td>1977</td>
<td>pathophysiology/histology</td>
<td>contact, flame</td>
<td>4</td>
<td>White Chester</td>
<td>.</td>
<td>17.5</td>
<td>.</td>
</tr>
<tr>
<td>Henze et al. (53)</td>
<td>1997</td>
<td>treatment/novel therapy</td>
<td>scald</td>
<td>18</td>
<td>Domestic-white</td>
<td>12</td>
<td>30</td>
<td>2.15</td>
</tr>
<tr>
<td>Hoekstra et al. (39)</td>
<td>1993</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>6</td>
<td>Yorkshire</td>
<td>14</td>
<td>35</td>
<td>.</td>
</tr>
<tr>
<td>Jander et al. (81)</td>
<td>2000</td>
<td>treatment/novel therapy</td>
<td>scald</td>
<td>10</td>
<td>Landrace</td>
<td>.</td>
<td>28.1</td>
<td>.</td>
</tr>
<tr>
<td>Kaczmarek et al. (174)</td>
<td>2001</td>
<td>diagnostic modalities</td>
<td>contact</td>
<td>8</td>
<td>.</td>
<td>.</td>
<td>25</td>
<td>.</td>
</tr>
<tr>
<td>Kim et al. (161)</td>
<td>2016</td>
<td>model development</td>
<td>contact</td>
<td>4</td>
<td>Red Duroc</td>
<td>.</td>
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<td>.</td>
</tr>
<tr>
<td>Liu et al. (175)</td>
<td>2008</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>3</td>
<td>Yorkshire</td>
<td>18</td>
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<tr>
<td>Lotter et al. (176)</td>
<td>2014</td>
<td>diagnostic modalities</td>
<td>contact</td>
<td>7</td>
<td>Mini-pig - Gottingen</td>
<td>39</td>
<td>22.6</td>
<td>1.29</td>
</tr>
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<td>Menon et al. (58)</td>
<td>2016</td>
<td>model development</td>
<td>contact</td>
<td>2</td>
<td>Great white</td>
<td>8</td>
<td>.</td>
<td>.</td>
</tr>
<tr>
<td>Moritz et al. (1)</td>
<td>1947</td>
<td>pathophysiology/histology</td>
<td>scald</td>
<td>.</td>
<td>.</td>
<td>.</td>
<td>9</td>
<td>.</td>
</tr>
<tr>
<td>Morykwas et al. (177)</td>
<td>1999</td>
<td>treatment/novel therapy</td>
<td>contact</td>
<td>25</td>
<td>White Chester</td>
<td>.</td>
<td>20</td>
<td>.</td>
</tr>
<tr>
<td>Nanney et al. (145)</td>
<td>1996</td>
<td>pathophysiology/histology</td>
<td>contact</td>
<td>4</td>
<td>Domestic- white</td>
<td>.</td>
<td>36</td>
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* Data unextractable, unclear, or not available
2.5.2 Quality assessment of selected publications

Individual quality scores for each study are given in Table 2-7 and summarised in Table 2-6. A grade of 2 (all criteria adequate) was achieved for ≥ 90% of the studies for checklist items describing; the title, abstract and introduction [1-4], the experimental procedures [7] and the discussion [18, 19]. Notably 29% of the studies had inadequate ethical statements [5] and 55% did not adequately mention or disclose funding sources/conflicts of interest [20]. None of the studies adequately (score of 2) explained how the number of burn replicates and/or animals for the study was determined by providing details of sample size calculations such as a power analysis [10].

Table 2-6 Summary of the quality of articles included in this review. Assessed using modified ARRIVE guidelines (items 11 and 14 excluded) where each item is scored 0-2. For item 17 (adverse events) 90% of studies were assessed as not applicable.

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2.5.3 Demographics

Demographic data is presented in Table 2-5 with comparisons between the studies shown in Figure 2-2. The exact terminology describing the site of burns varied. Burns located on the dorsal aspect (back, paravertebral, lateral thoracic and flanks) \((n = 38)\) were more common than burns on the ventral abdomen \((n = 3)\). Skin thickness (measured histologically) was only reported for a few studies \((n = 8)\), with an average of \(2.27 \pm 0.6\)mm, which is similar to skin thickness values reported for human adults \((94, 98)\). Studies whose primary aim was reported as development of a novel burn model accounted for 30% of all included studies \((n = 13)\). A high proportion of studies had the primary aim of investigating novel burn treatments \((n = 14)\) and a few studies reported evaluation of a diagnostic modality as the reason for the study \((n = 6)\).

![Figure 2-2 Demographics of studies. A. animal specifics, B. types of studies and C. breed of pigs.](image)

2.5.4 Burn creation

The different methods and materials for burn creation used by each study are reported in Table 2-8 and summarised in Figure 2-3. Individual burn sizes ranged from \(1.7 \)cm\(^2\) to approximately \(2000\)cm\(^2\) \((30\%\)TBSA\), with an average of \(16.5 \pm 18.1\)cm\(^2\) (excluding (53), which was exceptionally large). For \(n = 13\) studies the pressure with which the burning...
device was applied was reported. For the type of material used in the burn apparatus, there were \( n = 37 \) different contact burns included for analysis ((34 contact only studies), 2 (contact additional to another mechanism e.g. flame, scald) and 1 (contact only study where 2 separate materials were described)). There were 7 different materials described to create contact burns (Figure 2-3C), the majority used metal and a few (\( n = 2 \)) reported use of a plastic membrane. Brass was the most commonly used metal (43%), followed by aluminium (30%). The most common method for heating the materials used for contact burns was hot water (Figure 2-3D), which was used approximately 50% of the time.

**Figure 2-3 Burn creation details including:** A. burn injury mechanism, B. site of burns, C. type of material and D. method of heating material used for contact burn devices.
Table 2-8 Burn creation methodologies. If no burn size was reported, the average burn size was calculated from device dimensions.

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<th>Number of burns per pig</th>
<th>Average size of burn (cm²)</th>
<th>Burn Mechanism</th>
<th>Type of material (contact burns)</th>
<th>Heat source for burn device</th>
<th>Temperature (ºC)</th>
<th>Duration of exposure (sec)</th>
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<td>gas burner</td>
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<td>contact</td>
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<td>back</td>
<td>12</td>
<td>3.14</td>
<td>contact</td>
<td>stainless steel</td>
<td>.</td>
<td>176</td>
<td>6</td>
</tr>
<tr>
<td>Nanney et al. (145)</td>
<td>back</td>
<td>8</td>
<td>4.9</td>
<td>contact</td>
<td>.</td>
<td>.</td>
<td>56</td>
<td>10</td>
</tr>
<tr>
<td>Orgill et al. (178)</td>
<td>back</td>
<td>12</td>
<td>12.25</td>
<td>contact</td>
<td>aluminium</td>
<td>.</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td>Papp et al. (62)</td>
<td>ventral abdomen</td>
<td>8</td>
<td>16</td>
<td>contact</td>
<td>brass</td>
<td>heated water</td>
<td>100</td>
<td>3-12</td>
</tr>
<tr>
<td>Ragol et al. (179)</td>
<td>back</td>
<td>48</td>
<td>9</td>
<td>contact</td>
<td>brass</td>
<td>heated water</td>
<td>100</td>
<td>10-40</td>
</tr>
<tr>
<td>Rigal et al. (150)</td>
<td>flank</td>
<td>.</td>
<td>3.75</td>
<td>contact</td>
<td>aluminium</td>
<td>heated water</td>
<td>50-70</td>
<td>5-120</td>
</tr>
<tr>
<td>Schomacker et al. (128)</td>
<td>paravertebral</td>
<td>4</td>
<td>16</td>
<td>contact</td>
<td>brass</td>
<td>heated water</td>
<td>100</td>
<td>3-12</td>
</tr>
<tr>
<td>Sheu et al. (43)</td>
<td>back</td>
<td>8</td>
<td>10</td>
<td>contact</td>
<td>stainless steel</td>
<td>hot plate</td>
<td>80-110</td>
<td>20</td>
</tr>
<tr>
<td>Shi et al. (181)</td>
<td>back</td>
<td>20</td>
<td>3.14</td>
<td>contact</td>
<td>brass</td>
<td>heated water</td>
<td>100</td>
<td>45</td>
</tr>
<tr>
<td>Singer et al. (182)</td>
<td>back and flanks</td>
<td>20</td>
<td>6.25</td>
<td>contact</td>
<td>aluminium</td>
<td>heated water</td>
<td>100</td>
<td>20-30</td>
</tr>
<tr>
<td>Singer et al. (57)</td>
<td>back and flanks</td>
<td>18</td>
<td>6.25</td>
<td>contact</td>
<td>aluminium</td>
<td>heated water</td>
<td>50-100</td>
<td>10-30</td>
</tr>
<tr>
<td>Singer et al. (57)</td>
<td>back and flanks</td>
<td>20</td>
<td>6.25</td>
<td>contact</td>
<td>aluminium</td>
<td>heated water</td>
<td>70-90</td>
<td>20-30</td>
</tr>
<tr>
<td>Singer et al. (54)</td>
<td>back and flanks</td>
<td>2</td>
<td>8</td>
<td>contact</td>
<td>brass</td>
<td>heated water</td>
<td>100</td>
<td>30</td>
</tr>
<tr>
<td>Singh et al. (183)</td>
<td>back</td>
<td>.</td>
<td>7</td>
<td>contact</td>
<td>aluminium</td>
<td>heating pad</td>
<td>100</td>
<td>5-30</td>
</tr>
<tr>
<td>Todorovic et al. (184)</td>
<td>back</td>
<td>16</td>
<td>.</td>
<td>contact</td>
<td>.</td>
<td>electric cautery</td>
<td>150</td>
<td>10</td>
</tr>
<tr>
<td>Venter et al. (185)</td>
<td>back</td>
<td>4</td>
<td>.</td>
<td>scald</td>
<td>n/a</td>
<td>.</td>
<td>85</td>
<td>10</td>
</tr>
</tbody>
</table>

* Data unextractable, unclear, or not available
2.5.5 Outcome Measures

The burn conditions reported to create burns of equivalent depth or severity as evaluated histologically are provided in Table 2-9. There was extremely large variation between study reporting of when and how tissue damage was assessed. Time of biopsy ranged from immediately after burn creation up to 7 days post-burn. Many different staining methods were used, however, Hematoxylin and Eosin was the most common, reported by 70% of the studies. Several different histologic markers were used to determine injury depth, with cell necrosis and collagen injury the most common. Nearly half the studies reported only qualitative data for injury depth \((n = 19)\). Such wide variation in type and quality of tissue injury depth assessment affected the ability to compare study results. Due to the heterogeneity of the studies a meta-regression analysis was unable to be performed.

Where data was available, the average temperature and duration of exposure reported to cause superficial, SPT, DDPT and FT burns was analysed and is shown in Table 2-10. Due to the limited number of studies in each category, only DDPT burns were analysed for scald burns. Lower temperatures and shorter durations of exposure were reported for DDPT scald burns \((75–100^\circ C, 3–30 \text{ seconds})\) compared to DDPT contact burns \((65–190^\circ C, 6–60 \text{ seconds})\). Additionally, compared to scald burns, much greater variability between studies for the burn conditions reported to create DDPT contact burns was observed (Figure 2-4).

![Figure 2-4 Burn conditions (temperature and duration of exposure) reported for deep dermal partial thickness (DDPT) scald and contact burns. Error bars show the full range of data.](image-url)
<table>
<thead>
<tr>
<th>Author (ref)</th>
<th>Burn Mechanism</th>
<th>Burn conditions: Temperature (°C)/ Duration (sec)</th>
<th>Time of biopsy post-burn (hour)</th>
<th>Staining method</th>
<th>Histologic markers for injury depth</th>
<th>Quantitative data (Y/N)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brans et al. (37)</td>
<td>scald</td>
<td>. . . 80/30 . . .</td>
<td>1, 24, 48, 72</td>
<td>PAP, MT, EVG</td>
<td>cell necrosis, collagen denaturation</td>
<td>N</td>
</tr>
<tr>
<td>Brans et al. (37)</td>
<td>contact</td>
<td>. . . 170/20 . . .</td>
<td></td>
<td>PAP, MT, EVG</td>
<td>cell necrosis, collagen denaturation</td>
<td>N</td>
</tr>
<tr>
<td>Branski et al. (83)</td>
<td>contact</td>
<td>. . . 200/30 . . .</td>
<td>24, 48, 7days</td>
<td>H &amp; E, MT</td>
<td>collagen degeneration</td>
<td>N</td>
</tr>
<tr>
<td>Breuing et al. (166)</td>
<td>contact</td>
<td>. . . 85/10 . . .</td>
<td>96</td>
<td>H &amp; E</td>
<td>cell necrosis</td>
<td>Y</td>
</tr>
<tr>
<td>Brink et al. (163)</td>
<td>contact</td>
<td>190/1 . 190/3 . 190/21 . . .</td>
<td>1</td>
<td>H &amp; E, VE</td>
<td>coagulative necrosis</td>
<td>Y</td>
</tr>
<tr>
<td>Brown et al. (167)</td>
<td>contact</td>
<td>. . . 70/10 . . .</td>
<td>7d</td>
<td>.</td>
<td></td>
<td>N</td>
</tr>
<tr>
<td>Chvapil et al. (143)</td>
<td>contact</td>
<td>. . . 165/45 . . .</td>
<td></td>
<td>H &amp; E, MT</td>
<td>collagen injury</td>
<td>N</td>
</tr>
<tr>
<td>Brink et al. (163)</td>
<td>contact</td>
<td>. . . 200/30 . . .</td>
<td>24, 48, 72</td>
<td>PAP, EVG</td>
<td>collagen denaturation, cell viability</td>
<td>Y</td>
</tr>
<tr>
<td>Eldad et al. (170)</td>
<td>flame</td>
<td>. . . 1000/5 . . .</td>
<td>0, 48, 5d, 7days</td>
<td>H &amp; E</td>
<td>collagen architecture</td>
<td>N</td>
</tr>
<tr>
<td>Eldad et al. (171)</td>
<td>contact</td>
<td>. . . 65/60 . . .</td>
<td>4, 24, 8d</td>
<td>H &amp; E, VG</td>
<td>cell necrosis, collagen differentiation</td>
<td>N</td>
</tr>
<tr>
<td>Farhangkhoee et al.(59)</td>
<td>contact</td>
<td>. . . 100/20 . . .</td>
<td>1,12,24,36,48,72, 97</td>
<td>H &amp; E, Ki-67</td>
<td>cell necrosis, cell proliferation</td>
<td>Y</td>
</tr>
<tr>
<td>Gaines et al. (46)</td>
<td>contact</td>
<td>. . . 100/20 . . .</td>
<td>24, 8d</td>
<td>GT</td>
<td>collagen injury</td>
<td>Y</td>
</tr>
<tr>
<td>Glatter et al. (172)</td>
<td>contact</td>
<td>. . . 100/20 . . .</td>
<td>49</td>
<td>H &amp; E, LDH</td>
<td>cell viability</td>
<td>Y</td>
</tr>
<tr>
<td>Goans et al. (173)</td>
<td>contact</td>
<td>. . . 100/20 . . .</td>
<td>1</td>
<td>EVG</td>
<td>coagulative necrosis</td>
<td>Y</td>
</tr>
<tr>
<td>Gurfinkel et al.(80)</td>
<td>radiant heat</td>
<td>. . . 400/20 . . .</td>
<td>1</td>
<td>H &amp; E</td>
<td>denatured collagen, cell necrosis</td>
<td>Y</td>
</tr>
<tr>
<td>Gursu et al. (111)</td>
<td>contact</td>
<td>. . . 100/20 . . .</td>
<td>0, 6, 12, 24, 73</td>
<td>H &amp; E, VE</td>
<td>collagen injury, cell necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Henze et al. (53)</td>
<td>scald</td>
<td>. . . 75/25 . . .</td>
<td>4, 28, 52, 76</td>
<td>MTT</td>
<td>cell viability</td>
<td>Y</td>
</tr>
<tr>
<td>Hoekstra et al. (39)</td>
<td>contact</td>
<td>. . . 170/20 . . .</td>
<td>7d</td>
<td>PAP, EVG</td>
<td>granulation tissue, inflammation</td>
<td>N</td>
</tr>
<tr>
<td>Jandera et al. (81)</td>
<td>scald</td>
<td>. . . 83/11 . . .</td>
<td>1, 24, 7d</td>
<td>.</td>
<td>coagulative necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Kaczmarek et al.(174)</td>
<td>contact</td>
<td>. . . 80/30 . 100/30 . 1.5</td>
<td>.</td>
<td>.</td>
<td></td>
<td>Y</td>
</tr>
<tr>
<td>Kim et al. (161)</td>
<td>contact</td>
<td>. . . 200/40 . . .</td>
<td>0</td>
<td>H &amp; E</td>
<td>collagen architecture</td>
<td>Y</td>
</tr>
<tr>
<td>Liu et al. (175)</td>
<td>contact</td>
<td>. . . 100/20 . . .</td>
<td>24</td>
<td>H &amp; E</td>
<td>coagulative necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Lotter et al. (176)</td>
<td>contact</td>
<td>100/1-3 . 100/12 . 100/30 . 100/60 . 4</td>
<td>.</td>
<td>MT</td>
<td>collagen injury</td>
<td>Y</td>
</tr>
<tr>
<td>Menon et al. (58)</td>
<td>contact</td>
<td>. . . 90/20 . . .</td>
<td>.</td>
<td>H &amp; E, PAS,</td>
<td>collagen injury/vessel</td>
<td>N</td>
</tr>
<tr>
<td>Moritz et al. (1)</td>
<td>scald</td>
<td>. . . 50/300, 75/5 . . .</td>
<td>24, 49</td>
<td>H &amp; E</td>
<td>epidermal necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Author (ref)</td>
<td>Burn Mechanism</td>
<td>Burn conditions: Temperature (°C)/ Duration (sec)</td>
<td>Time of biopsy post-burn (hour)</td>
<td>Staining method</td>
<td>Histologic markers for injury depth</td>
<td>Quantitative data (Y/N)</td>
</tr>
<tr>
<td>----------------------</td>
<td>----------------</td>
<td>-----------------------------------------------------</td>
<td>---------------------------------</td>
<td>-----------------</td>
<td>-------------------------------------</td>
<td>------------------------</td>
</tr>
<tr>
<td>Morykwas et al. (177)</td>
<td>contact</td>
<td>60/5,70 100/3</td>
<td>24, 48, 72, 8d</td>
<td>H &amp; E, GT</td>
<td>cell necrosis, collagen</td>
<td>Y</td>
</tr>
<tr>
<td>Nanney et al. (145)</td>
<td>contact</td>
<td>100/6</td>
<td>24, 48, 74, 97</td>
<td>Vimentin</td>
<td>cell viability</td>
<td>Y</td>
</tr>
<tr>
<td>Orgill et al. (178)</td>
<td>contact</td>
<td>176/6</td>
<td>100/45</td>
<td>H &amp; E, MT</td>
<td>collagen denaturation</td>
<td>N</td>
</tr>
<tr>
<td>Papp et al. (82)</td>
<td>contact</td>
<td>100/1</td>
<td>2, 24, 48, 73</td>
<td>H &amp; E</td>
<td>vascular patency, cell necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Ragol et al. (179)</td>
<td>contact</td>
<td>100/3</td>
<td>8, 32, 104</td>
<td>H &amp; E</td>
<td>Collagen, other elements</td>
<td>Y</td>
</tr>
<tr>
<td>Rigal et al. (180)</td>
<td>contact</td>
<td>60/30</td>
<td>1, 2, 3, 5, 6, 9, 10, 18</td>
<td>H &amp; E, MT</td>
<td>cell necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Schomacker et al. (128)</td>
<td>contact</td>
<td>100/10 100/15</td>
<td>1, 24, 49</td>
<td>LDH</td>
<td>cell viability</td>
<td>Y</td>
</tr>
<tr>
<td>Sheu et al. (43)</td>
<td>contact</td>
<td>80/20</td>
<td>1, 2, 3, 5, 6, 9, 10, 18</td>
<td>H &amp; E, MT</td>
<td>collagen injury</td>
<td>Y</td>
</tr>
<tr>
<td>Shi et al. (181)</td>
<td>contact</td>
<td>100/45</td>
<td>4, 5, 6, 8, 8d</td>
<td>H &amp; E</td>
<td>cell necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Singer et al. (182)</td>
<td>contact</td>
<td>80/20</td>
<td>2</td>
<td>EVG</td>
<td>collagen injury</td>
<td>Y</td>
</tr>
<tr>
<td>Singer et al. (55)</td>
<td>contact</td>
<td>70/20 80/20 90/20 100/30</td>
<td>0,5</td>
<td>H &amp; E</td>
<td>cell necrosis, collagen</td>
<td>Y</td>
</tr>
<tr>
<td>Singer et al. (57)</td>
<td>contact</td>
<td>80/20</td>
<td>1, 2, 3, 5, 6, 9, 10, 18</td>
<td>H &amp; E, MT</td>
<td>collagen injury</td>
<td>Y</td>
</tr>
<tr>
<td>Singer et al. (54)</td>
<td>contact</td>
<td>80/20</td>
<td>4, 5, 6, 8, 8d</td>
<td>H &amp; E</td>
<td>cell necrosis</td>
<td>N</td>
</tr>
<tr>
<td>Singh et al. (183)</td>
<td>contact</td>
<td>100/30</td>
<td>72</td>
<td>H &amp; E</td>
<td>collagen injury</td>
<td>Y</td>
</tr>
<tr>
<td>Todorovic et al. (184)</td>
<td>contact</td>
<td>150/10</td>
<td>0,5</td>
<td>H &amp; E</td>
<td>not specified</td>
<td>Y</td>
</tr>
<tr>
<td>Venter et al. (185)</td>
<td>scald</td>
<td>85/10</td>
<td>3, 24</td>
<td>H &amp; E, MT</td>
<td>cellular necrosis, collagen</td>
<td>Y</td>
</tr>
</tbody>
</table>

Data unextractable, unclear, or not available; H & E: Hematoxylin and Eosin; PAP: Papanicolaou stain; EVG: Elastica van Gieson; MT: Masson's Trichrome; GT: Gomori's Trichrome; LDH: Lactate dehydrogenase; PCNA: Proliferating cell nuclear antigen; VG: Van Gieson; VE: Verhoff's Elastica; MTT: Modified (4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazoliumbromide.
Table 2-10 The temperatures and durations of exposure reported to create different severity burns.

<table>
<thead>
<tr>
<th>Burn severity</th>
<th>Injury Mechanism</th>
<th>Average time (sec)</th>
<th>Time range (sec)</th>
<th>Average temperature (°C)</th>
<th>Temperature range (°C)</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>Superficial</td>
<td>Contact</td>
<td>11</td>
<td>1–30</td>
<td>102</td>
<td>60–190</td>
<td>5</td>
</tr>
<tr>
<td>SPT</td>
<td>Contact</td>
<td>12</td>
<td>3–20</td>
<td>109</td>
<td>70–190</td>
<td>15</td>
</tr>
<tr>
<td>DDPT</td>
<td>Contact</td>
<td>23</td>
<td>6–60</td>
<td>111</td>
<td>65–190</td>
<td>17</td>
</tr>
<tr>
<td>DDPT</td>
<td>Scald</td>
<td>14</td>
<td>3–30</td>
<td>83</td>
<td>75–100</td>
<td>6</td>
</tr>
<tr>
<td>FT</td>
<td>Contact</td>
<td>33</td>
<td>12–60</td>
<td>131</td>
<td>100–200</td>
<td>13</td>
</tr>
</tbody>
</table>

SPT, superficial partial thickness; DDPT, deep dermal partial thickness; FT, full thickness

2.6 Discussion

2.6.1 Quality of studies

Generally the quality of the studies included for review was adequate. The majority had high quality scores for the key methods items, research objectives were clear and interpretation of the results and their relevance to human biology was adequately addressed. It is concerning that one third of the studies had inadequate ethical statements, although most of those studies were published decades ago. Encouragingly, all studies published after 1996 had adequate ethics statements. Notably, a small percentage of studies lacked any details (score of 0) for basic method items describing procedures to reduce bias, animal welfare assessments and statistical methods used for analysis. While poor quality scores in these items did not prevent inclusion of studies in this review, inconsistencies in reporting can have an effect on the ability to evaluate the strength of their conclusions. Interestingly, none of the studies provided details of sample size calculations (such as a power analysis) for the number of replicates required for each burn condition tested. Applying the ARRIVE guidelines to all areas of bioscience research using animals is encouraged (164). As such, in addition to upholding the principles of the 3 R’s, providing details of sample size calculations would enhance the quality and comprehensiveness of reporting for experimental porcine burn models.

2.6.2 Study types and demographics

As anticipated, common study types included burn model development and pathophysiology/histology studies. Interestingly, there was a high proportion of studies whose primary aim was investigation of novel burn treatments or diagnostic modalities.
Given the large number of porcine burn models in the literature, it is surprising that new models are continually developed for these types of studies, rather than optimising existing methods. By comprehensively analysing existing porcine burn models and evaluating how burn conditions relate to injury severity, it is anticipated this review will serve as resource for researchers and minimising future animal use.

The demographics of the pigs in the studies was similar. Whilst the use of mini-pigs in burns research has gained popularity in recent years, the overwhelming majority of studies reported using white domestic pigs (e.g. Large Whites, Yorkshires and Chesters). The majority of studies used juvenile pigs, which are large enough to manage multiple procedures and anaesthetics, but not so big as to be overly difficult to handle. Most studies reported creating multiple individual burns, with an average of 12 moderately sized (17cm²) burns per pig. A general lack of consistency in reporting all animal specifics was observed (e.g. species, sex, age, weight), however, similar animal demographics between most studies allowed for comparison.

It is commonly acknowledged that models using scald as a mechanism of injury are more technically challenging and may increase the risk of unintentional burn injury to researchers compared to contact burn models (44, 65). Therefore, it is not surprising that the majority of porcine burn models reviewed here were contact burn models. Not only were there more studies, but the range of temperatures and contact times used to generate contact burns was much greater than for scald burns. Only one out of the five scald models included reported a comprehensive range of burn conditions, however, this study by Moritz and Henriques (1) did not provide quantitative histologic details for depth of injury to the dermis, making comparisons for severity of dermal injury limited. By excluding the Moritz and Henriques study (1), the range of water temperatures evaluated became much narrower (75–85°C). These findings highlight a gap in knowledge regarding estimating the severity of injury to the dermis which results from exposure to moderate temperature water (50–75°C) for relatively long durations (>30 seconds), such as with immersion scalds, and high temperature water (>85°C) for short durations (<10 seconds) seen with accidental spill/splash injuries. Further experimental work in this area is recommended.

2.6.3 Comparison of the burn conditions for burns of equivalent severity

2.6.3.1 Contact burns

Though a large number of contact burn studies were included, collation of the data was
difficult due to the heterogeneity of materials used in the burn devices, for example, brass, aluminium, stainless steel, iron and water with a plastic membrane. The thermal properties of each material are unique, with heat capacity and ability to transfer heat to the tissue differing widely. Additionally, the method for heating burn devices and the pressure with which they were applied to the skin also differed, which made comparisons between models challenging. Therefore, a quantitative analysis with meta-regression was unable to be performed. Regardless, there was an apparent association between burn severity and average duration of exposure, with burn severity increasing as the average duration of exposure to the device increased. Average contact times of 11, 23 and 33 seconds were reported for superficial, DDPT and FT contact burns, respectively.

The average of the burn conditions reported to cause DDPT contact burns was 111°C for 23 seconds. The usefulness of this result as a guide for future model development studies is problematic due to the wide variety of materials used to create contact burns. As suggested by Singh et al. (183), consideration of the correlation between the actual amount of heat transferred and the severity of injury would enhance standardisation of future studies. While beyond the scope of this paper, the data collated here provides a valuable resource for further mathematical modelling studies considering variables such as specific heat capacity, density and thermal conductivity of the material used, and the area of contact. Comparing the actual amount of heat delivered by different materials for the equivalent severity of injury would be of enormous benefit to both researchers and regulators.

2.6.3.2 Scald burns

As all of the included scald burn models used hot water to create burns, it was anticipated the data could be collated to determine a heat dose to damage relationship. However, only five scald burn models could be included in the analysis. Unfortunately, the study of Moritz and Henriques (1) did not report histological data for depth of dermal injury to validate the burn conditions reported to cause a DDPT burn. Additionally, a further two studies reported only qualitative results for burn injury severity. Due to the limited number of studies and the narrow range of burn conditions tested, no evaluation of the burn conditions reported to cause a SPT or FT scald burn could be made. Including all scald studies, the average burn conditions required to cause a DDPT burn were 83°C water for 14 seconds, or excluding (1), 81°C water applied for 19 seconds. This result is comparable to a recent study investigating hot beverage spills using experimental data from skin tissue surrogates and numerical simulations (79). The authors estimated that a mid-dermal injury would result from
a spill with 82°C water where saturated clothing was left in contact with the skin for 30 seconds.

### 2.6.3.3 Radiant heat and flame burns

Only one study used a radiant heat model, and while there were two flame burn models included, only one reported the temperature and duration of exposure, therefore, data collation of the studies was not possible for these burn injury mechanisms.

### 2.6.4 Contact verses scald burns

Comparison between the conditions reported to cause scald and contact burns of equivalent severity was only possible for DDPT burns. A wide disparity was apparent with substantially lower temperatures and shorter exposure times reported for scald burns (83°C for 14 seconds) compared to contact burns (111°C for 23 seconds). Given the relatively poor thermal conductivity of water compared to metals this result may appear surprising. However, the specific heat capacity of water is much higher than that of the metals described, for example, ten times higher than brass. Therefore, the amount of thermal energy stored in water heated to 83°C is greater than that of a brass rod heated to the same temperature. This review demonstrates the importance of the effect of mechanism of injury on burn injury severity. We recommend that the burn conditions established by porcine contact burn models to create certain depth of injury should not be used by regulators to guide scald burn prevention strategies.

The limitations that made comparisons between studies for the burn conditions difficult included different classifications used to describe tissue injury severity, evaluations of burn injury depth made at different times post-burn, different stains used on the tissue and different markers for cell injury. Several studies had incomplete information for burn creation methods and materials used. Additionally, for some studies where information regarding burn conditions and burn depth or severity was not clearly reported or in graphs, subjective interpretation of the data was necessary. We also acknowledge the possibility of selection bias given the exclusion of articles where full-text was not available in English. This review was limited to studies where depth of damage was determined histologically within 7 days of burn creation. Whether mid-dermal and deep dermal damage as evaluated histologically is representative of a burn which clinically would take longer than 3 weeks to heal is not investigated here. Future reviews of porcine burn models where the outcome measure of time to healing is investigated would be beneficial.
2.7 Conclusion

This novel systematic review with 42 included studies provides a comprehensive overview of porcine burn models used in burn injury research. In summary:

- Overall, the quality of the included papers was modest when assessed using the ARRIVE guidelines.
- Despite availability of several well-validated models, researchers continue to develop new models for use in studies investigating burn treatments or diagnostics.
- The average burn conditions reported to cause a DDPT scald burn (83°C for 14 seconds) are different to contact burns (111°C for 23 seconds). The clinical implication of this is that equivalent severity injuries maybe sustained at lower temperatures and/or shorter durations with scald burns.
- It remains difficult to estimate the severity of injury to the dermis from exposure to moderate temperature water (50–75°C), as seen with hot tap water immersion scalds, and more research is required in this area.
- The heterogeneity of the different burn apparatus materials for contact burn models limits the identification of burn conditions required to create burns of a pre-defined depth. Further calculations or modelling to estimate the amount of thermal energy delivered by each apparatus are necessary.
- The key significance of this review is that it was performed systematically, allowing a novel grouped analysis of results from different studies to evaluate the relationship between burn temperature, duration of exposure and burn severity.

2.8 Acknowledgments

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Conflicts of interest: All the authors have no conflicts of interest.
2.9 Chapter Conclusion

The systematic review presented in this chapter demonstrates that although many well-validated porcine burn models have been published, it remains difficult to estimate the burn conditions required to cause a deep dermal scald injury. Interestingly, although only a few scald burn studies could be included for analysis, the average burn conditions reported for a DDPT scald injury (83°C for 14 seconds) were of much greater intensity (higher temperature and longer duration) than suggested by Moritz and Henriques data (1). Additionally, this review confirmed that mechanism of injury has an important effect on burn injury severity, with equivalent severity injuries sustained at lower temperatures and/or shorter durations with scalds compared to contact burns. In short, together with the literature review presented in chapter 1, this comprehensive systematic review provides clear evidence supporting the necessity for a re-evaluation of the time and temperature thresholds for deep scald injuries.

The findings from the systematic review presented in this chapter were also used to inform the development of the novel porcine scald model presented in the following chapter (chapter 3). The modest quality results observed for many of the articles reviewed here was considered when developing and reporting on the animal experiments for this research project. For example, emphasis was placed on following the ARRIVE guidelines when reporting sample size calculations, methods to reduce bias and statistical methods. Additionally, information collected here from previous porcine experimental studies was used to inform histopathological investigations regarding the timing of biopsies and staining methodologies used to assess pathological changes in burned tissue (presented in chapter 3 and Appendix A).
Chapter 3 Porcine scald model development

This chapter is based on a publication in the journal PLoS ONE

Citation:

3.1 Chapter foreword

This chapter describes the methodological approach used to investigate the burn conditions (temperature and duration of exposure) required to sustain scald injuries, similar to those encountered by children. As explained in the preceding chapters (chapters 1 and 2), none of the existing porcine scald burn models described by the literature were considered suitable for these thermal injury investigations. The purpose of this chapter is to describe in detail the novel, consistent and reliable porcine scald injury model developed for this research project.
Development of a Consistent and Reproducible Porcine Scald Burn Model

3.2 Abstract

There are very few porcine burn models that replicate scald injuries similar to those encountered by children. We have developed a robust porcine burn model capable of creating reproducible scald burns for a wide range of burn conditions. The study was conducted with juvenile Large White pigs, creating replicates of burn combinations; 50°C for 1, 2, 5 and 10 minutes and 60°C, 70°C, 80°C and 90°C for 5 seconds. Visual wound examination, biopsies and Laser Doppler Imaging were performed at 1, 24 hours and at 3 and 7 days post-burn. A consistent water temperature was maintained within the scald device for long durations (49.8 ± 0.1°C when set at 50°C). The macroscopic and histologic appearance was consistent between replicates of burn conditions. For 50°C water, 10 minute duration burns showed significantly deeper tissue injury than all shorter durations at 24 hours post-burn (p ≤ 0.0001), with damage seen to increase until day 3 post-burn. For 5 second duration burns, by day 7 post-burn the 80°C and 90°C scalds had damage detected significantly deeper in the tissue than the 70°C scalds (p ≤ 0.001). A reliable and safe model of porcine scald burn injury has been successfully developed. The novel apparatus with continually refreshed water improves consistency of scald creation for long exposure times. This model allows the pathophysiology of scald burn wound creation and progression to be examined.

3.3 Introduction

Burns are a common and potentially devastating cause of injury in childhood. Scald burns as a mechanism of thermal injury are of particular importance as globally they are still the most commonly treated paediatric burn injury (10). In Australia approximately 2000 children will be hospitalised each year with burns, with over half of these admissions being for scalds (hot liquid or steam) (12, 13, 15). Many experimental animal models of burn injury are reported in the literature (reviewed in (65, 160)) using rats, mice, rabbits, guinea-pigs and pigs with various mechanisms of thermal injury, such as, scald, contact, radiant heat and flame. Given the anatomical and physiological similarities between porcine and human skin (44, 67, 69, 70), pigs are considered by many to be the optimal species for cutaneous thermal injury investigations (1, 65, 69, 70). A porcine scald burn model is arguably the
model of most translational relevance to paediatric burns as it is recognised clinically that the pathophysiology of scald burns is different to that of contact burns (37, 56). However, whilst there are many published porcine contact burn models (2, 39, 43, 44, 46, 54, 83, 161), there are few porcine models that replicate a true scald injury such as those encountered by children.

Existing porcine scald burn models described by Henze et al. (53) and Radke et al. (186) used an immersion technique to create one large scald burn covering 30% of the total body surface area (TBSA). Although water is maintained at a consistent temperature for long durations, these studies are limited by testing only one temperature/duration combination per animal and the difficult logistics of suspending a large animal over a water bath. Brans et al. (37) utilised a bottomless applicator to create several small burns on the flank of the same animal. However, using a single application of water at the desired temperature (80 °C for 10, 20, 30, 40 seconds) is limited, as water rapidly cools in the device once removed from the heat source. To test for a broad range of burn conditions, the ability to create several small uniform wounds on the same animal is desirable. Additionally, maintaining a consistent water temperature within the scald device improves the consistency and reproducibility of scald burn creation.

Scald spill/splash injuries occur from exposure to very hot liquids for only a short skin contact duration, for example, hot beverage scalds. However, immersion scalds are also a frequent mechanism of scald burn injury, occurring as a result of exposure to more moderate temperature liquids for longer durations, for example, bath water scalds. The aim of this study was to develop a porcine scald model that could be used to examine clinically relevant burn conditions. Based on the design of Moritz and Henriques published studies (1, 33) a novel scalding device was developed. The apparatus allows for continuous refreshment of the hot water within the device and a more consistent water temperature for long durations. Additionally, in order to better understand how scald burns progress in the acute peri-burn period, it was desirable to create a burn area large enough such that multiple, well-spaced sequential biopsies could be taken from the same burn. Therefore, while Moritz and Henriques (1) created numerous (approximately 34) very small burns (4.9cm²) on each pig, our intent was to develop a model where several (eight) moderately sized burns (16cm²) could be created on the same pig.

Several techniques were used in this study to analyse, compare and evaluate the wounds,
validating the model’s ability to create reproducible and consistent burns. Evaluations included subdermal temperature monitoring, quantitative histological analysis of tissue injury, and Laser Doppler Imaging (LDI). Importantly, sequential analysis of the burns over 7 days allows for assessment of how the burn wounds evolve in the early post-burn period.

The primary focus of this manuscript is to provide a comprehensive description of a novel method for reproducible scald burn generation, exploring the extent of tissue injury resulting from exposure to a range of clinically relevant burn conditions. The model’s unique ability to replicate burn injury for a broad range of scald burn conditions is highly desirable and has wide application for researchers investigating novel burn therapies or diagnostics, as well as those investigating the pathology of burn injury.

3.4 Materials and methods

3.4.1 Ethics Statement

All methods conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition) published by the Australian National Health and Medical Research Council. Ethics approval was obtained from the University of Queensland Animal Ethics Committee (Approval numbers: QCMRI/RCH/326/12/QCMRI/NHMRC and QCMRI/446/15/QCHF). All procedures were performed under a general anaesthetic and all efforts were made to minimise suffering.

3.4.2 Animals

Female Large White juvenile pigs of 27 kg (approximately 12 weeks of age) were used for the study. Pigs were delivered to the animal house 7 days prior to commencing the experiment to allow for acclimatisation. Animals were given a standard pellet diet and free access to water. On days prior to procedures requiring administration of anaesthesia the pigs were fasted overnight.

3.4.3 General Anaesthetic and Monitoring

Anaesthesia was induced intramuscularly with Ketamine 13 mg/kg (Ketamine 100 mg/ml Ceva™, Glenorie, NSW, Australia) and 2 mg/kg Xylazine (Ilodium Xylazil 100 mg/ml, Ilodium™, Troy laboratories Pty Ltd, NSW, Australia). A size 4 laryngeal mask airway was inserted and anaesthesia was maintained at a surgical plane with 1%–2.5% Isoflurane (Attane, Bayer, Australia Ltd) in oxygen (1–3 litre/min flow). Buprenorphine 0.01 mg/kg (Temgesic® 0.3 mg/ml
Reckitt Benckiser Healthcare, Slough, UK) was administered intramuscularly peri and post-operatively to provide analgesia. A 24 gauge IV cannula was placed in the lateral auricular vein and intravenous fluids (0.9%NaCl) were administered at a rate of 5ml/kg/hr throughout the procedure. A transdermal Fentanyl 50µg/hr patch (Durogesic®50, Janssen-Cilag Pty Ltd, North Ryde, Australia) was applied for additional post-operative analgesia. Animals underwent the same general anaesthetic regime at each biopsy collection time point. On day 7 the animals were euthanased with 15ml of sodium pentobarbitone (Lethabar®TM, Virbac Pty Ltd, Penrith, NSW, Australia) administered intravenously.

3.4.4 Wound creation

3.4.4.1 Device

The materials used to make the scald burn device can be readily sourced from hardware and plumbing stores available in most countries. The device consisted of a 60mm diameter stainless steel pipe with foam insulation tubing fitted around the outside to allow an operator to hold the device safely (Figure 3-1). The insulation material was folded back onto itself on the bottom edge to create a padded lip which was placed onto the animal (there was no direct contact between the metal pipe and the skin surface). The aperture at the bottom of the device for direct contact between water and the skin surface was approximately 50mm. To provide uniform downward pressure, two weighted lead rings (1kg when taped together) were placed on top of the device. Plastic tubing (4mm diameter) sitting 20mm above the bottom of the device was connected to vacuum suction, ensuring a consistent level of water was maintained within the device at all times.
Figure 3-1 Scald creation device. Consists of metal pipe covered with insulation tubing which is folded onto itself at the bottom and a clear plastic vacuum suction tube. A. ‘padded lip’ bottom of device, B. side view of device with weighted rings placed on top, C. Schematic of device showing inflow and outflow of hot water.

A temperature controlled water bath (Grant Instruments, Cambridge, UK) was used to maintain the water at a constant, tightly regulated temperature. A digital 5411 Fluke® thermometer (Fluke Australia Pty Ltd., North Melbourne, Australia) was used to measure the water temperature within the scald device. Water was pumped into the scald device at a rate of 1.3 litres per minute using a submersible aquarium pump (set at 4.5 Volts) and vacuum suctioned out at an equivalent rate. Using the maximum temperature values recorded from initial experiments and trials of the system external to the pig as a guide, we found that setting the water bath to 3°C higher than the desired target temperature (for temperatures ≥ 70°C) ensured the water in the device at the time of scald creation was most precise. The heat loss within the system for lower temperature burns was minimal and with experience we found setting the water bath to 0.3°C higher than the target temperature (for temperatures ≤ 60°C) was desirable. Pre-warming the device immediately prior to scalding and insulating the inflow tubing was also beneficial.

3.4.4.2 Site of burn

In total eight burns were created on the thoracic paravertebral area of each animal (four on each side), with 2–3 centimetres between burns. Two replicate burns were created for each of four time/temperature combinations per pig. To account for any variation in healing due to anatomical location (187), burn positions on one side of the animal were assigned
randomly and replicate burns (on the other flank) were assigned to the opposite anatomical location.

3.4.5 Scald burn creation

Preparation of the burn sites included clipping the hair on the back and flanks, and marking the sites for burning using the device as a template. Protective clear plastic sheets were taped in place (Figure 3-2A, 1) to cover the immediate surrounding area and reduce the risk of an unintentional burn. Immediately prior to scalding, the device and tubing was pre-warmed for at least 1 minute by running the system external to the pig. After pre-warming, the device was placed perpendicular to the skin surface and weighted rings were placed on top to provide uniform downward pressure for a leak proof seal. The same operator supported the device with one hand and controlled the hot water inflow tube with their other hand (Figure 3-2C). At the end of the exposure the inflow of hot water was halted by pinching the tube, the whole scald device was lifted off the animal and any excess water was quickly mopped up with a towel. A range of burn conditions were tested; 50°C for 1, 2, 5 and 10 minutes; and 60, 70, 80 and 90°C for 5 seconds.

After wound assessments (detailed below), all burns were dressed with Melolin™ and Fixomull® (Smith & Nephew, North Ryde, NSW, Australia) which was changed at each time point. To further protect the burn area, custom made garments (2) were fitted.

3.4.6 Temperature monitoring

Body temperature was measured at regular intervals using a rectal digital thermometer (MC-110B, Omron Corporation, Japan). Subdermal temperature probes (K type thermocouples, Radiospares Components Pty LTD., Smithfield, Australia) were used to monitor the temperature within the skin during scald creation, using our previously published method (157, 188, 189) (Figure 3-2A, 2). The depth of the probe in the subcutaneous tissue was measured using ultrasound ((LOGIQ e R7 series with a 22 MHz hockey stick probe (GE Healthcare, Parramatta, NSW, Australia)) (Figure 3-2B). Temperatures were logged by the
Fluke thermometer every second once the heated water was applied or every 10 seconds for burns of 10 minute duration. Logging of subdermal temperatures was continued for at least 60 seconds after the heat source was removed, or until a maximum temperature was reached. The external skin surface temperature was measured at a neighbouring site, using a separate temperature probe (Figure 3-2A, 3).

3.4.7 Wound Assessment

After burn creation and at each biopsy time point, wounds were examined visually and the total area of the burns were assessed using a Visitrak™ device (Smith & Nephew, Australia) (2). Digital photographs were also taken of each wound using a Canon EOS 300D digital SLR camera (Canon Australia, North Ryde, Sydney).

3.4.7.1 Histological investigation

Full thickness 8mm skin biopsies were obtained from all of the burns at 1 and 24 hours and days 3 and 7 post-burn. A sample of normal skin from each animal was also taken at each time point. To minimise any local inflammatory effects post-biopsy, sequential biopsies were taken from separate representative areas. Each biopsy sample was fixed in 10% neutral buffered formalin for 24 hours and embedded in paraffin. Routine Haematoxylin and Eosin (H&E) staining was performed on 5µm thick paraffin sections. Sections were digitally captured using a Nikon EP600 microscope (Nikon Instruments Inc, USA) fitted with a Spot RT slider cooled CCD camera (SPOT Imaging Solutions™, Sterling Heights, USA) and scored by an examiner blinded to burn conditions. Quantitative measurements (in mm) for the minimum, maximum and average thickness of the dermis and depth of dermal damage over the entire section were electronically calculated using Image Pro Plus v.5.1 software (Media Cybernetics, Silver Spring, USA). All dermal elements were evaluated for damage, with a line of damage representative of the deepest tissue injury detected for each section. The average depth of this line was used to calculate results presented here as % injury to dermis by depth (average depth of damage to dermis divided by the average total depth of the dermis). Markers of tissue injury for H&E sections included blocked vessels (140), endothelial cell injury (57), adnexal necrosis (61), infiltration of inflammatory cells and dilation of lymphatic vessels (46) (Figure 3-3).
Figure 3-3 Examples of tissue injury markers identified using H & E staining. Images from A. 90°C for 5 sec and B. 50°C for 10 min scald at 72 hours post-burn. Thick black arrow indicates free red blood cells, thin black arrow indicates blocked vessels, black triangles show where the epidermis has lost adherence, asterisk indicates adnexal necrosis and open black arrow points to inflammatory cell infiltration seen; within glands, perivascular and as a thick band within the tissue of the dermis.
3.4.8 Laser Doppler Imaging

Laser Doppler Imaging (LDI) was performed at each time point to assess blood flow to the wound. The LDI scanner (Moor LDI 2, Moor Instruments, Devon, UK) was positioned perpendicularly over the flank of the animal such that all four burns were imaged simultaneously (Figure 3-2D). The average distance between the scanner head and the skin surface was 37cm. Scans were performed using the settings for a large scanning area with high resolution (256 X 256 pixels) and a slow scan speed of 4ms/pixel. Analysis of images using Moor LDI Image Processing v2.4 (Moor Instruments Ltd, Devon, UK) was performed by defining the wound area as a “region of interest” (ROI) for each burn and calculating the average perfusion units (PU) per wound. Individual biopsy sites within each burn were also traced as an ROI and excluded from calculations. For each flank one perilesional ROI of normal skin was measured as a control. The mean blood perfusion ratio of burned skin (B) to normal (N) perilesional skin was calculated as described previously by others (126). A B/N ratio < 1 indicates less blood perfusion than normal skin, = 1 designates same perfusion as normal, > 1 shows increased perfusion from normal. Burn areas showing no macroscopically visible signs of injury (at the time of scanning) were not reviewed and blood perfusion was considered the same as normal skin (B/N = 1). Higher than normal blood flow indicates a more superficial burn and low flow indicates a full thickness burn, whereas deep partial thickness dermal burns may have a blood flow similar to or lower than normal skin.

3.4.9 Data analysis

The statistical analysis was performed using GraphPad Prism V6 (GraphPad Software, Inc. California, USA) and SPSS 22 (IBM Corporation, Armonk, N.Y, USA). Results are reported as mean ± standard error of the mean (SEM) unless otherwise stated. Analysis of the LDI ratios was performed using a method previously reported (126). A two way analysis of variance (ANOVA) was performed at each observation time point with a Tukey’s multiple comparison test to compare the burn conditions for significant differences (p < 0.05). A Spearman’s correlation was used to calculate an intra-observer reliability rating for histological scoring of dermal damage, with significance set at p = 0.01 (2 tailed).

3.5 Results

A total of 40 individual burns were included for analysis. There were n ≥ 4 replicates of each burn temperature/time combination (50ºC for 1, 2, 5 and 10 minutes and 60ºC, 70ºC, 80ºC...
and 90°C for 5 seconds). The average burn size was 16.36 ± 1.59cm², with a length of 4.79 ± 0.32cm and a width of 4.52 ± 0.35cm. The pig’s average weight on scald creation day was 27kg and the estimated total body surface area of the burns was 2% (190). The average thickness of the dermis on scald creation day was evaluated with histology to be 2.29 ± 0.45mm.

3.5.1 Validation of water temperature in scald device

Temperatures logged from the probe within the scald device fluctuated in the first 5–10 seconds before stabilising, at which point a more consistent and accurate reading of the water temperature was obtained. The temperature of the water in the scald device when the input water temperature was 50°C (from the water bath) was 49.8°C ± 0.1°C (Figure 3-4A). The maximum temperature recorded for water in the device for the 5 second duration scalds is shown in Figure 3-4B.

![Figure 3-4](image)

**Figure 3-4 Temperature of water in the device during scald creation.** A. 50°C water for 5 and 10 min exposure (n = 8). B. 5 second duration exposures showing the maximum temperature recorded for water in the scald device for each target temperature (n ≥ 4 for each temperature). Bars are mean ± SEM.

3.5.2 Consistency of scald burn creation

3.5.2.1 Macroscopic

All burns had a uniform circular shape with a consistent red colour throughout the burn area immediately after creation. By 1 hour post-burn the more superficial burns were beginning to lose their intensity, becoming less distinct with a mottled white and red appearance, while the distinctive deep red/hyperaemic appearance of the more intermediate severity burns had intensified (Figure 3-5). At 1 hour, the 90°C for 5 sec scalds were observed to have a
red hyperaemic ring around the burn with a uniform blanched white appearance, indicative of a more severe burn.

![Image of wound appearances 1 hour post-burn.][1]

**Figure 3-5 Wound appearance 1 hour post-burn.** Intensity of colour varies with burn severity.

### 3.5.2.2 Microscopic

The macroscopic and histologic appearance of wounds was consistent between replicates of the same burn condition at each time point (Figure 3-6 and Figure 3-7). By 7 days post-burn there was no microscopic evidence of cell damage for the 50°C for 1 and 2 minute burns or the 60°C for 5 second burns. All other burn conditions showed some degree of tissue damage at 7 days post-burn. A gradient of tissue injury was observed with more damage to the cells in the superficial dermis compared to the deeper dermal tissue below. Results for the line representing depth of damage are the deepest level to where damage was observed i.e. the endpoint of this graduated tissue damage.

For 50°C water, by 24 hours post-burn, the 10 minute duration group showed significantly deeper tissue injury than all other exposure durations ($p \leq 0.0001$), with damage depth observed to increase up to day 3. For 5 second duration burns by day 7, the 80°C and 90°C scalds had significantly deeper dermal damage than the 70°C scalds ($p \leq 0.001$). Extent of damage was also observed to increase over time post-burn for both the 80°C and 90°C scalds, this was statistically significant for 90°C for 5 second scalds, showing deeper...
damage at day 3 and 7 post-burn compared to 1 hour post-burn ($p \leq 0.02$). Conversely, for 70°C scalds there was no increasing extent of damage over time post-burn and although it was not statistically significant, the extent of damage on day 7 was less than the damage at earlier time points.

![Figure 3-6 Comparison of macroscopic and histologic appearance for 4 replicates of 50°C/10 min burns at 24 hours post-burn shows consistency in appearance and depth of injury. Dotted black line represents the deepest level where tissue injury was observed. Black rectangle indicates location of high power image (40X). Thin black arrows point to examples of tissue injury (congestion of vessels).]

A blinded re-test ($n = 30$) of randomly selected sections was performed by the same examiner, with a high intra-observer reliability rating ($r = 0.87$).
Figure 3-7 Depth of dermis damaged as a percentage of total dermal depth, for each condition and time point. A. 50ºC burns, B. 5 second exposure burns. Bars are mean ± SEM. * significantly deeper than other burn conditions at the same time point (p ≤ 0.001).

3.5.3 Subdermal temperature

To compensate for variations in the baseline subdermal temperatures from individual animals (34.2 ± 0.9ºC), the results were calculated as change in temperature from starting temperature. The magnitude of subdermal temperature change was greater for burns of low temperature/long duration than for higher temperature/shorter duration burns. Maximum subdermal temperatures for each burn condition are given in Table 3-1. For short duration burns (5 seconds) the subdermal temperature continued to rise over the first 60 seconds post-burn, in contrast to the long duration burns (2–10 minutes) where the temperatures decreased immediately after heat source removal. The average distance from the bottom of the dermis to the subdermal temperature probe was 2.6 ± 0.6mm, measured with ultrasound in vivo.

Table 3-1 Subdermal temperatures for each burn condition.

<table>
<thead>
<tr>
<th>Burn condition (temperature(ºC)/duration (sec))</th>
<th>Maximum subdermal temperature ± SEM (ºC)</th>
<th>Maximum change in subdermal temperature ± SEM (ºC)</th>
<th>Highest subdermal temperature recorded (ºC)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50/60</td>
<td>38.6 ± 1.1</td>
<td>4.4 ± 1.3</td>
<td>44.0</td>
</tr>
<tr>
<td>50/120</td>
<td>41.4 ± 1</td>
<td>7.0 ± 1</td>
<td>43.6</td>
</tr>
<tr>
<td>50/300</td>
<td>41.8 ± 0.9</td>
<td>7.4 ± 1.1</td>
<td>45.5</td>
</tr>
<tr>
<td>50/600</td>
<td>43.9 ± 1</td>
<td>9.8 ± 1.3</td>
<td>47.8</td>
</tr>
<tr>
<td>60/5</td>
<td>35.9 ± 0.4</td>
<td>2.7 ± 0.8</td>
<td>36.5</td>
</tr>
<tr>
<td>70/5</td>
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<td>2.4 ± 0.2</td>
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</tr>
<tr>
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<td>37.2 ± 1.1</td>
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<td>39.0</td>
</tr>
<tr>
<td>90/5</td>
<td>39.1 ± 0.2</td>
<td>4.8 ± 0.6</td>
<td>39.5</td>
</tr>
</tbody>
</table>
3.5.4 Laser Doppler Imaging

The mean perfusion units (PU) of normal tissue (perilesional unburned skin) was 238.4 ± 74.1. There was no significant difference in the mean PU of normal skin for scans performed on different days. The mean blood perfusion ratio of burned skin (B) to normal (N) perilesional skin for all burn conditions tested at each time point is shown in Figure 3-8. At 1 hour post-burn there was no significant difference in the B/N ratio between un-burned normal skin and any of the burns. For 50°C water, at 24 hours post-burn, the 5 and 10 minute duration burns showed significantly higher B/N ratios than normal (p ≤ 0.05). However, by day 3 and 7 post-burn the only 50°C burn showing a higher than normal B/N ratio was the 10 minute duration burn. For water applied for 5 seconds, at 24 hours all the burns had significantly higher B/N ratios than normal (p ≤ 0.05). By day 3 and 7 all burns, except for the 60°C (which had no visible burn), showed significantly higher than normal B/N ratios. None of the burn conditions tested had a B/N ratio significantly lower than unburned skin at any of the scan time points. For both the 80°C and 90°C burns there was a strong trend of increasing perfusion with time post-burn, with B/N ratios at day 7 significantly higher than at 24 hours and day 3 (p ≤ 0.04, p ≤ 0.03).

![Image of Figure 3-8](image_url)

**Figure 3-8** Laser Doppler Imaging Burn to Normal (B/N) ratio for different burn conditions. A. 50°C scalds and B. 5 second scalds at different time points post-burn. Bars are mean ± SEM. *significantly different to normal (B/N = 1, p ≤ 0.05).

3.6 Discussion

An effective burn model must have reliable and consistent tissue injury. One of the major advantages of a scald burn model is that the hot water is able to uniformly cover the entire area of exposed skin despite any irregularities in the skin surface. It has been reported for porcine contact burn models that less than optimal close contact between the burn infliction...
device (e.g. metal bar) and the skin surface (44, 62) can result in variability and inconsistencies in uniformity of depth of tissue injury. Researchers have attempted to overcome this effect by utilising a bottomless bottle sealed with malleable plastic wrap (2, 87, 88) which allows increased flexibility of the device to conform to the curved body contour of the flank. However, even with this method, small irregularities in the skin surface or air pockets trapped between the device and the skin (191) may lead to variability in the uniformity of contact achieved. The consistency in the visual surface appearance of the scald burns immediately after creation confirms the uniformity of contact between the heat source (water) and the skin. In addition to consistency of surface appearance, it is arguably the uniformity of the depth of tissue injury (evaluated microscopically) for a specified burn condition which is required to verify the reliability of a burn model. The deepest level to where observable tissue damage was detected was consistent between replicates of the same burn condition with this model.

By taking biopsies at different time points in the early post-burn period (over 7 days) a more accurate assessment of the depth of tissue injury can be conducted. The dynamic phenomenon of burn wound progression has been well documented since it was described by Jackson (23). As burn wounds evolve over time, caution is advised in interpreting early post-burn histological appearance. Here, by day 3 post-burn the full extent of the damage to the dermis was better recognised, as has been reported by others (23, 192). For burns of moderate severity (80°C and 90°C for 5 second and 50°C for 10 minutes) there was a general trend of increasing depth of damage observed from 1 hour post-burn to day 3 post-burn, when the deepest level of tissue damage was recorded. This indicates that biopsies taken in the first 24 hours post-burn may underestimate the extent of tissue damage for some burn conditions, and should be interpreted with caution. Whether this delay in detecting the full extent of damage is as a result of the inability to identify functional cell damage with H&E staining or due to burn wound progression is unclear. Future work using alternative staining techniques, similar to those investigated by others using a contact burn model (61), will be performed to better assess cell viability.

The 70°C for 5 second scalds were observed to show a similar depth of dermal damage from 1 hour to day 3 post-burn. However, by day 7 these burns revealed histological evidence of healing and repair with regeneration of the epidermis and reduced depth of tissue damage. Comparatively, at day 7 the 80°C and 90°C for 5 second scalds had indicators of both viability (epithelial proliferation, appearance of organising granulation
tissue) and continued deep dermal tissue damage (inflammatory cell infiltration and endothelial cell damage), indicating that these burns were more severe. Whether these burns are representative of a severe deep partial thickness injury which takes longer than 3 weeks to heal and requires grafting is not established here, as time to healing for the different wound conditions was not investigated. Results from this study will be used to guide future investigations where a small range of burn conditions will be followed for longer than 7 days and time to healing established, so that histological indicators of damage can be correlated to clinical wound healing.

Clinically, it is recognised that deep dermal partial thickness burns require more extensive treatment than superficial dermal partial thickness injuries. Consequentially, there is significant interest in being able to predict the threshold temperature required to cause injury to the deep dermal tissue from a spill/splash exposure. While results presented here are for histological assessment of tissue damage and not time to healing, it is still meaningful to relate these findings to previous studies predicting burn injury depth in human skin. Here, a 5 second exposure time was considered to be representative of a spill/splash injury. Histological assessment at day 7 indicates that water ≥ 80°C resulted in injury to the deep dermal tissue. This injury threshold is at the lower end of those previously stated for numerical modelling (64, 78) and simulation (104) studies, where predictions for burn injury depth in human skin are reported. Additionally, our results concur with a recent study which quantified scald burns from hot beverage spills using both experimental data (obtained from skin and skin tissue surrogates) and numerical simulations (79, 193). The authors reported a spill temperature of 82°C as the threshold for mid-dermal injury. We acknowledge, as do others (78, 79, 193), that experimental and numerical modelling studies represent an idealised situation and their limitations should be considered when comparing to the reality of human burns. The circumstances of each accidental spill/splash injury are unique and threshold for injury estimates reported here should be considered as a guide, rather than absolute.

Porcine burn models for investigating novel diagnostics and therapeutics are developed by creating a burn of pre-defined injury severity (2, 43, 46, 57, 83), which allows for assessment of healing progression. Presented here was an experimental model with emphasis placed on exploring the extent of tissue injury resulting from exposure to different scald burn conditions, similar to those encountered by children. As such, the deepest line of damage was representative of the deepest point at which any evidence for tissue damage was
observed. For most of these scald burns, a gradient of tissue injury was observed (most apparent by day 3 and 7 post-burn), with more cells damaged in the superficial dermal tissue compared with the deeper dermis. For high temperature contact burns, a clear line of demarcation separates viable and non-viable areas within the dermis (37). No such precise delineation was evident here and we suggest a gradient of injury is more prominent for scald burns of moderate severity (e.g. 80°C and 90°C for 5 seconds, 50°C for 10 minutes). Whether this is due to the differing pathophysiology of scald burns compared to contact burns, the magnitude of the heat dose applied, or differences in the regenerative capacity of cells within the dermis becoming more apparent at these time points, is unknown.

The results shown in this study also illustrate the importance of comprehensive histological evaluation of depth of damage to the dermis for validation. Burn prevention strategies, such as legislation of hot water delivered to sanitary fixtures (e.g. bathrooms) to be no greater than 50°C, are based on Moritz and Henriques (1, 194) studies where the threshold for damage was described as full thickness destruction of the epidermis. However, as noted by others (48, 49), results from their studies have been and continue to be erroneously translated and extrapolated such that full thickness damage to the epidermis (trans-epidermal necrosis) is incorrectly equated to a more severe full thickness burn. For example, scald burn prevention literature states that exposure to 50°C water for 5 minutes causes a full thickness burn (195) and it only takes 5–6 seconds for water at 60°C to cause a full thickness burn (196, 197). Here, by day 7 the only 50°C burn showing any histological indicators for deep dermal damage was the 10 minute exposure. By day 3 post-burn the 50°C for 5 minute duration burns showed significant healing and were barely visible macroscopically with only minimal dermal damage detected histologically. Similarly, for the 60°C for 5 second burn, by day 3 burns were not macroscopically visible and no dermal damage could be detected histologically. In future we will investigate a broader range of burn conditions, which will enable us to develop a clearer understanding of the relationship between burn depth and heat dose.

Measuring the subdermal temperature changes during thermal injury allows us to improve understanding of how heat is conducted through living skin. Quantitative analysis regarding heat conduction in skin using our experimental data and mathematical modelling is explored elsewhere (189). In this study, the subdermal temperature profiles of the 60–90°C for 5 second burns were similar, with a relatively small average increase in temperature from 2.7–4.8°C and temperatures continued to rise after heat source removal. Conversely, we
observed for longer durations of exposure (2–10 minutes) the magnitude of subdermal temperature change was much greater although the applied water temperature was lower (50°C). Interestingly, the change in subdermal temperature for the 50°C for 1 minute burns and the 90°C for 5 second burns was similar (4.4°C and 4.8°C respectively), however, these burns demonstrated markedly different tissue damage, with the 50°C for 1 minute burn displaying no macroscopic or microscopic signs of injury at 24 hours post-burn, whereas the 90°C for 5 second burns had microscopic evidence of damage to the deep dermis and a blanched white appearance indicative of a severe burn. Using clinically relevant burn conditions and relating temperature changes within the skin to tissue damage has significant clinical applications and requires further investigation. Future improvements to our model may include investigating how heat is transferred through the different layers of the skin by including multiple temperature measurements at different depths.

One of the most commonly cited limitations of a scald infliction method is the concern of increased risk of burn injury to the researchers when compared to contact burn models (44, 65). We acknowledge there is potential for accidental burn injuries to investigators or the animal with our model, however, no such incidents occurred. One key design feature unique to our model to safeguard against operator and unintentional animal injury was that only a small volume of water was in the device (just sufficient to cover the skin) at any given time. Furthermore our model reduces excess handling of the hot water by pumping the heated water straight from the water bath to the device rather than transfer via another heat source (37, 81). The authors recommend additional personal protective equipment of long sleeved insulated rubber gloves and eyewear be worn for all high temperature scalds.

It is well documented in human studies that the reliability of LDI scanning in the immediate (early) post-burn period is questionable (132, 137). Studies by Stetinsky et al. (137) report that the perfusion status of both SPT and DDPT burns were similarly low in the acute post-burn period (48 hours). They suggested that localised oedema may compress the vascular supply to the area and thus low perfusion reflects disruption rather than actual destruction of the microvasculature. Our results using a porcine model support this contention. Perfusion trends in the early (1 hour) post-burn period were generally lower than at all later time points, with similar results recorded for all burns despite their differing severity (as determined by histopathology). However, in this study increased perfusion was observed by as early as 24 hours post-burn. Interestingly, none of the burn conditions tested displayed significantly lower than normal perfusion, including burns with evidence of damage into the deep dermis.
(measured histologically). Fourman et al. (60, 127) questions the reliability of LDI imaging to assess accuracy of predicting burn scarring (127) and viability of the zone of ischemia (60) using a porcine contact burn model. They observed difficulty in interpreting intermediate and low perfusion measurements, arguing that while LDI was able to reliably measure the hyperemic response of superficial dermal injuries, it was less capable of differentiating deeper dermal porcine injuries. Results presented here support these claims, with a strong trend of increased perfusion from 24 hours post-burn for burns displaying superficial dermal damage. Additionally, burns considered to be of moderate severity (mid–dermal or with evidence of deep dermal damage e.g. 80–90°C for 5 seconds, 50°C for 10 minutes) which we anticipated would show reduced blood flow (low B/N ratios) actually displayed significantly higher perfusion than normal by 24 hours. Nonetheless, for these burns the B/N ratio continued to increase to day 7 post-burn indicating some delay in reaching maximum perfusion compared to the other burn conditions tested in this study. It is possible higher than expected perfusion results may have occurred as a result of biopsies causing additional inflammation and pathological disruption to the burn injury area despite these areas being excluded from analysis. Future planned studies where healing outcomes are assessed will include LDI scans of burns where no prior biopsy samples have been taken.

Secondary outcome measures including analysis of severity of tissue injury with histology and LDI are presented here to demonstrate the models ability to create consistent burns and are intended to serve as a guide only when considering the exact heat dose required to create a burn of pre-defined injury severity for testing new burn treatments. Ultimately, we plan to use this model to better understand the relationship between burn injury severity and heat dose by investigating a broader range of burn conditions and including outcome measures such as time to healing.

In conclusion, whilst many investigators recognise both the relevance and advantages of a using a scald burn model over a contact burn model for certain studies, until now it has often been cited as too technically challenging and posing an unacceptable risk of injury to investigators. Presented here is a reliable and safe method of scald burn creation in a porcine model. Additionally, the novel apparatus with continually refreshed water improves consistency of scald creation for long duration exposures.
3.7 Acknowledgements

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3.8 Chapter Conclusion

The porcine scald model, presented in this chapter, established a reliable method to create and evaluate scalds representative of human scald injuries.

The methodology for the porcine scald model has been published and all experimental chapters in this thesis follow this scheme of scald creation, data collection and data analysis. The multitude of data and tissue samples collected during both the creation of the burn model and whilst conducting animal experiments enabled development of other studies, which form additional bodies of work produced for this thesis. In the following two chapters, these studies will be addressed separately and include: an examination of heat conduction in skin (chapter 4), and evaluating changes in skin thickness measurements with excision and biopsy processing procedures (chapter 5). Each of these experimental chapters will include further elucidation of methodological details where required.

In addition to the 12 week old pigs described in this chapter, a pilot study involving four younger (5 week old) pigs was also conducted. For the younger pigs, the methodology of scald creation was as described in this chapter, however, a smaller diameter scald device was used. The chapter that follows analyses the subdermal temperature results obtained from both these 12 week old and 5 week old pigs.
Chapter 4  Development of a mathematical model to examine heat conduction in skin

This chapter is based on a publication in the *International Journal of Heat and Mass Transfer*.

Citation:

4.1 Chapter foreword

Understanding how heat is conducted through the skin is a vital first step towards examining the relationship between heat conduction and tissue damage. This chapter examines the thermal properties of skin, describing development of a mathematical model used to interpret data from the initial experimental animal studies presented in this thesis. The temperature profile of the skin during scald injury creation for different burn conditions was measured using subdermal temperature probes (protocol described in chapter 3). With mathematical modelling, this data was used to consider how the thermal properties of skin depend on burn duration, burn temperature, and skin thickness. It is important to consider these variables because thermal injuries may occur under many different conditions.

Note, for this chapter, due to journal requirements, where appropriate for equations and figures, time in seconds is abbreviated to s.
Quantifying the role of burn temperature, burn duration and skin thickness in an *in vivo* animal skin model of heat conduction

4.2 Abstract

To determine the extent to which heat conduction through skin is affected by skin thickness, burn temperature, and burn duration, we perform a suite of experiments using an *in vivo* porcine model. Fourteen different burn conditions are considered, and each burn condition is replicated at least four times, giving a total of sixty four individual experimental burns. The subdermal temperature within the skin is recorded as a function of time during each experiment. To quantitatively interpret the experimental data, we develop an exact solution of a simplified, depth-averaged, heat equation. Calibrating this solution to the experimental data provide estimates of the effective thermal diffusivity of the skin, $\alpha$, and the effective thermal loss rate, $k$. Estimates of $\alpha$ and $k$ are obtained for the fourteen different, clinically relevant, burn conditions. Overall, we find $\alpha = 0.03 \pm 0.02 \text{mm}^2/\text{s}$, and is approximately independent of the burn duration, burn temperature, and skin thickness ($H$). This estimate implies that the time required for thermal energy to diffuse vertically down, through the skin of thicker ($H = 2.27 \text{mm}$) and thinner ($H = 1.40 \text{mm}$) skinned animals is approximately 170 and 70 seconds, respectively. We find that $k = 0.002 \pm 0.002/\text{s}$. In summary, our results provide contemporary estimates for the thermal properties of *in vivo* porcine skin, which has broad application to heat transfer modelling investigations of thermal injury prevention and thermal therapy studies.

Nomenclature

$t$ time (s)

$x$ position (mm)

$T(t)$ average temperature in the tissue layer (°C)

$\Delta T(t)$ change in temperature in the tissue layer relative to the initial temperature (°C)

$T_w$ temperature of the water (°C)

$T_s$ surface temperature of the skin (°C)
4.3 Introduction

Scald burns from accidental exposure to hot liquids are the most common cause of thermal injury in children (10). These burns are extremely painful, may require prolonged treatment, and can cause scarring (113). Fundamental knowledge of how heat is conducted through skin is essential to inform the development of burn prevention strategies. One way to improve our understanding of heat conduction in skin is to apply a mathematical model to interpret an experimental data set.

Quality experimental data examining heat conduction in living skin are rare. Most experimental research in this area is conducted with pig skin, because pig skin is anatomically and physiologically similar to human skin (1, 65, 67, 69, 70). The most extensive experimental data set exploring heat conduction in skin is the 1947 study by Henriques and Moritz (194). They excised skin tissue from pigs and estimated the thermal conductivity, $\alpha$, of the different layers of non-living, \textit{ex vivo}, skin. Henriques and Moritz (194) also estimated the caloric uptake of pig skin \textit{in vivo} when a metal heat source was applied to the surface of the skin and used this information to provide an estimate of the thermal conductivity for \textit{in vivo} conditions. Their estimates of $\alpha$ for the pig dermis is 0.096mm$^2$/s and 0.108–0.288mm$^2$/s for \textit{ex vivo} and \textit{in vivo} conditions, respectively. More recent \textit{ex vivo} studies using excised pig skin range from using very thick, 5mm tissue sections including fat (198), to using extremely thin 30µm sections of epithelial cells (199), and these studies report values for $\alpha$ of 0.11mm$^2$/s and 0.029mm$^2$/s, respectively. Unfortunately, it is unclear
how these kinds of measurements, using different portions of the skin layers in non-living tissue, translate to the more complex situation in living tissue.

Mathematical models of heat transfer in skin are traditionally based on the Pennes bioheat equation (75, 200, 201). This model is an extension of the standard linear heat equation (202, 203) with an additional source term that accounts for thermal energy loss (200, 201). Often the source term is used to represent loss to the blood supply, which is known as perfusion (75, 200, 201). Studies that directly combine mathematical modelling with comprehensive *in vivo* experimental data sets are scarce. Most previous mathematical models have been parameterised by extrapolating experimental data from existing literature (109, 204-207), or from experimental data sets that describe a single burn condition only (108, 208). In contrast, here we use a mathematical model to directly interpret thermal measurements in a suite of *in vivo* experiments. In these experiments, we vary several key features such as skin thickness, burn duration, and burn temperature. This approach provides us with a unique opportunity to explore whether the thermal diffusivity and thermal loss depend on skin thickness, burn duration or burn temperature. These details have not been addressed in previous combined studies that make use of both experimental measurements and mathematical models (209).

Examining the relationship between temperature, burn duration and injury severity is essential for guiding scald burn injury prevention standards. Accidental and non-accidental hot tap water scalds may result from exposure to moderate temperature water for long durations (210, 211). One key scald prevention approach is regulating the temperature of hot water delivered by bathroom taps in domestic dwellings, which in many countries is legislated to be no greater than 48°C (212, 213) or 50°C (214). For this reason, we focus our experiments on moderate temperature burn conditions (50–60°C) over relatively long durations (60–600 seconds). Since we deal with a heat transfer process through a relatively thin layer of skin, and our experiments do not measure spatial differences in temperature within the thin layer of skin, we interpret the experiments using a simplified, depth-averaged, mathematical model that neglects spatial variations in temperature (215, 216). This allows us to develop an exact, closed-form solution of the simplified mathematical model. We calibrate this solution to data from various experimental conditions, providing multiple opportunities to estimate $\alpha$ and the loss rate, $k$. Our combined experimental and modelling study provides valuable, quantitative information about heat conduction in living skin that cannot be obtained by extrapolating from previous *ex vivo* experiments.
4.4 Materials and Methods

4.4.1 Animal Experiments

A porcine burn model is chosen because of the anatomical and physiological similarities to human skin (1, 65, 67, 69, 70). Juvenile pigs have a skin thickness of 2–2.3mm (12 weeks old) (43, 53) and 1–1.3mm (5 weeks old) (1), which closely approximates the skin thickness of a human adult and a human child (94), respectively. All methods conform to the Australian National Health and Medical Research Council published code of practice for animal research. Ethics approval was obtained from the University of Queensland’s animal ethics committee (QCMRI/446/15/QCHF), and all animals were treated in a humane manner. Seven female Large White juvenile pigs, weighing 27kg and aged 12 weeks, and two female Large White weaner pigs, weighing 10kg and aged 5 weeks, were used. Pigs were delivered to the animal house seven days prior to commencing the experiment to allow for acclimatisation. Animals were given a standard pellet diet and free access to water, with fasting overnight prior to anaesthesia.

4.4.1.1 Study protocol

Scald burns were created on the backs of pigs whilst they were under a general anaesthetic. Anaesthesia was induced with Ketamine 13mg/kg (Ketamine 100mg/ml Ceva™, Glenorie, NSW, Australia) and 1mg/kg Xylazine (Ilium Xylazil 100mg/ml, Ilium™, Troy laboratories Pty Ltd, NSW, Australia) and maintained with Isoflurane (Attane, Bayer, Australia Ltd). Buprenorphine 0.01mg/kg (Temgesic® 0.3mg/ml Reckitt Benckiser Healthcare, Slough, UK) was administered peri-operatively to provide analgesia and a transdermal Fentanyl 50µg/hr patch (Durogesic®50, Janssen-Cilag Pty Ltd, North Ryde, Australia) was applied for post-operative analgesia. The hair on the back and flanks was clipped prior to scald creation. On day seven (endpoint of main experiment) the animals were euthanased with 15ml of sodium pentobarbitone (Lethabarb™, Virbac Pty Ltd, NSW, Australia).

Eight burns were created on the paravertebral region of each pig, with four burns per side. The burn injury was created by applying a purpose made scald device (Figure 4-1). Two different diameter devices were used for the two different age pigs, with a 50mm diameter for the older animals and a 30mm diameter device for the younger animals, as the smaller animals have a reduced surface area available for burn creation. Pre-heated water was pumped into the scald device and vacuum suctioned out at an equivalent rate, ensuring a constant level of water within the device at all times. We used a water bath (Grant
Instruments, Cambridge, UK) to maintain the water at a constant temperature. Water temperature within the scald device was recorded using a digital 54II Fluke® thermometer (Fluke Australia Pty Ltd., North Melbourne, Australia).

**Figure 4-1 Scald creation device and subdermal temperature monitoring.** The scald creation device consists of a metal pipe covered with insulation tubing and a suction tube. A. Schematic showing inflow and outflow of hot water in the 50mm diameter scald creation device. B. Photograph showing an example of creating a burn where: (I) hot water inflow; (II) suction of water out of the device; (III) insertion point of the subdermal temperature probe; and (IV) shows the Fluke thermometer, where the upper number is the subdermal temperature and the lower number is the temperature of water in the scald creation device.

### 4.4.1.2 Temperature monitoring

For each experiment the subdermal temperature probe was inserted using a previously described technique (157, 188). The subdermal temperature probe was used to monitor the temperature within the skin during each experiment. Briefly, prior to scalding, a 14 gauge 2.1mm by 45mm cannula was inserted obliquely from outside the marked wound area and advanced under the dermis until the tip was in the centre of the burn area. The stylet was removed and a type K thermocouple (Radiospares Components Pty Ltd., Smithfield, Australia) was inserted and taped into position. A digital 54II Fluke thermometer automatically logged temperature measurements for the initial subdermal temperature, denoted $T(0)$, and then every second after the heated water was applied for the shorter burn duration experiments (60–120 seconds), or every 10 seconds for the longer burn duration experiments (300–600 seconds). Subdermal temperature logging was continued.
for at least 60 seconds after the heat source was removed, or until a maximum, steady, subdermal temperature was reached. The temperature of the external skin surface was measured at a different site on the same animal using a separate temperature probe.

In the 12 week old pigs the following burn conditions were tested: temperature of the water, $T_w = 50, 55, \text{ and } 60^\circ\text{C}$, for burn durations of $t_d = 60$ and $120$ seconds, and then $T_w = 50$ and $55^\circ\text{C}$, for $t_d = 300$ and $600$ seconds. In the 5 week old pigs, with thinner skin, four burn conditions were tested: $T_w = 50^\circ\text{C}$ for $t_d = 60, 120, 300$ and $600$ seconds.

Overall, the experimental programme involved performing fourteen different groups of burns. Each of the fourteen different burn conditions was replicated at least four times, giving a total of sixty four individual burns. The burn conditions included here for analysis were specifically chosen as part of another study to examine clinically mid-deep dermal burns. As such, for $T_w = 60^\circ\text{C}$, a burn duration of $t_d = 120$ seconds led to a severe full thickness burn injury and additional testing for longer durations was not required. For experiments involving the 5 week old pigs, the range of burn conditions tested was limited, based on ethical approval for younger animals.

4.4.2 Mathematical Modelling

Theoretical studies of heat conduction in biological tissues often invoke Pennes bioheat equation (75, 201, 205, 217, 218). This partial differential equation describes the variation of temperature in a biological tissue as a function of position and time. Since we measured the temperature at one location within the layer of skin, we do not report any spatial differences in temperature throughout the skin layer. Furthermore, we find that the initial subdermal temperature, $T(0)$, and the external surface temperature of the skin are very similar, and so there is no initial variation in temperature with depth. This observation, combined with the fact that these experiments focus on heat transfer through a relatively thin tissue, motivate us to consider a simplified depth-averaged mathematical model that neglects spatial variations in temperature (215, 216). Accordingly, the experiments are modelled using an ordinary differential equation instead of a partial differential equation.

Assuming the average temperature in the tissue is $T(t)$, the mathematical model governing the dynamics of the experiments can be written as,
Equation 1

\[
\frac{dT(t)}{dt} = \frac{\alpha}{H^2} \left[T_{\text{burn}}(t) - T(t)\right] - k(T(t) - T_{\text{ref}}),
\]

where \( t \) is time, \( \alpha \) is the thermal diffusivity, \( H \) is the skin thickness, \( T_{\text{burn}}(t) \) is the temperature of the scald burn applied at the surface, and \( k \) is the thermal loss rate. The thermal loss is taken to be proportional to the difference between the average temperature within the tissue and some reference temperature, \( T_{\text{ref}} \).

To solve Equation 1 we must specify \( T_{\text{burn}}(t) \) and \( T_{\text{ref}} \). For all experiments we consider, we model the applied scald burn using

Equation 2

\[
T_{\text{burn}}(t) = T_w \quad t < t_d,
\]
\[
T_{\text{burn}}(t) = T_s \quad t > t_d,
\]

where \( T_w \) is the temperature of the water in the scald creation device, \( t_d \) is the duration of the burn, and \( T_s \) is the surface temperature of the skin. The choice for the form of \( T_{\text{burn}}(t) \) in Equation 2 exactly replicates the applied surface temperature for the initial period, \( t < t_d \). Setting \( T_{\text{burn}}(t) = T_s \) in the latter period, \( t > t_d \), is an approximation. Our rational for using this approximation is that it correctly models the expected long time behaviour, \( T(t) = T_s \) as \( t \to \infty \).

We must also specify the reference temperature for the loss term in Equation 1. Since the initial measured subdermal temperature is very similar to the measured surface temperature, \( T(0) \approx T_s \), we assume that \( T_{\text{ref}} = T_s \). This means that when the heat source is applied and the average temperature in the skin layer increases above the initial temperature, there will be some loss of thermal energy. This loss could represent perfusion into the blood supply (75, 201, 205, 217, 218) or to another area of the tissue.

With these assumptions, and writing \( \lambda = (\alpha T_w / H^2 + k T_s) / (\alpha / H^2 + k) \) for notational convenience, the solution of Equation 1 can be written as
To apply Equation 3 to our experiments we assume that $T_w, T_s, t_d$ and $H$ are either measurable, or are specified as a part of the experimental design. Therefore, the only unknown parameters in the model are $\alpha$ and $k$. To estimate these two parameters we calibrate Equation 3 to provide the best match to the experimental measurements of the subdermal temperature. We use MATLAB’s lsqcurvefit routine (219) to provide a least-squares estimate of $\alpha$ and $k$.

4.5 Results and Discussion

4.5.1 Experimental results

Data from a total of sixty four burns are used, with at least four replicates for each burn condition. The average thickness of the dermis, $H$, measured microscopically, is $2.27 \pm 0.25\text{mm}$ in the 12 week old animals, and $1.40 \pm 0.14\text{mm}$ in the 5 week old animals. Based on our experience, we assume that the position of the subdermal probe is directly beneath the dermis, thus the depth of the probe is approximately $H$. The average initial subdermal temperature $T_{(0)}$, prior to scalding is $34.2 \pm 0.8\degree\text{C}$, which is very similar to the average initial external surface temperature of the skin, $T_s = 33.7 \pm 0.9\degree\text{C}$. This suggests that, at the beginning of the experiment, there is no spatial variation in temperature with depth.

Results for subdermal temperatures are shown in Figure 4-2. These results are reported in terms of the average temperature profiles that are obtained by averaging the experimental data across each experimental replicate for each burn condition considered. Overall, the trend is for the subdermal temperature to increase most rapidly when the heat source is first applied. The subdermal temperature then continues to increase, but at a slower rate, for the remainder of the exposure. Once the heat source is removed the temperature decreases. Some of the longer duration burn conditions eventually approach a steady subdermal temperature profile, which has also been reported by others (198). For example, the
experiments with $t_d = 600$ seconds and $T_w = 50^\circ C$ (Figure 4-2D) appear to approach a steady subdermal temperature of approximately $44^\circ C$, after approximately 400 seconds. This indicates that there must be some loss in the system otherwise the steady subdermal temperature would eventually approach $T_w = 50^\circ C$. We observe a slightly different trend for the shorter duration burns (60–120 seconds) since the subdermal temperatures do not appear to reach a steady temperature within the timescale of these shorter experiments.

**Figure 4-2 Averaged subdermal temperature profiles for different burn conditions.** Results in A-D correspond to $T_w = 60$, 55 and 50°C (12 week old pigs), and $T_w = 50^\circ C$ (5 week old pigs), respectively. Different burn durations include: $t_d = 60$ (black); 120 (red); 300 (green); and 600 seconds (blue). Data points correspond to the sample mean, and the error bars indicate the sample standard error, $\sigma/(n-1)$.

Similar to others (81, 174, 194), our results in Figure 4-2 indicate some variation between different experimental replicates of the same burn condition. We quantify this variation in terms of the sample standard error. There are two main causes of this variability. Firstly, these are *in vivo* animal experiments, and results are subject to the natural variations in skin structure between animals, and natural variations between different anatomical locations on
the same animal. Secondly, although placement of the subdermal probe is performed by the same person, some variation in the probe placement is unavoidable. In particular, it is possible that some probes were placed deeper into the subcutaneous tissue beneath the dermis than others. However, our study is strengthened by including multiple replicates for the same burn condition on different animals at different anatomical locations.

Comparing subdermal temperature profiles in thicker and thinner skinned animals (Figure 4-2 C–D), indicates that the same burn condition leads to different outcomes. In general, we see the same burn condition applied to a thinner skinned animal leads to higher subdermal temperatures compared to the thicker skinned animals. To further explore these differences we show the magnitude of change in subdermal temperature, $\Delta T(t) = T(t) - T(0)$, for some of these experiments in Figure 4-3. For the same burn conditions we observe that the magnitude and rate of increase in subdermal temperature is greatest for thinner skin. To the best of our knowledge, this is the first in vivo experimental study demonstrating directly comparable data describing heat conduction in skin of different thickness.

![Figure 4-3](image)

**Figure 4-3 Average change in subdermal temperature for 50°C scalds.** Data for both 5 week (red) and 12 week (blue) animals are given. Results in A. correspond to $t_d = 60$ seconds, and results in B. correspond to $t_d = 120$ seconds.

### 4.5.2 Modelling results

All experimental measurements for each burn condition were repeated at least four times. The model calibration procedure is applied to each set of replicate data to produce several estimates of $\alpha$ and $k$ for each burn condition. Results in Figure 4-4 compare some experimental data to the solution of the calibrated mathematical model. While the calibrated mathematical model does not capture every detail of the experimental data, the key features
are broadly replicated.

**Figure 4-4** Comparison of experimental data for a single experimental replicate to the solution of the calibrated mathematical model for four different burn conditions. Results in A–D correspond to \( T_w = 60, 55, 50^\circ\text{C} \) (12 week old animals) and \( T_w = 50^\circ\text{C} \) (5 week old animals), respectively. In all cases the experiments correspond to \( t_d = 120 \) seconds. Each plot compares the in vivo experimental data (orange) with the solution of the calibrated mathematical model (green).

Results summarizing estimates of \( \alpha \) and \( k \) for the fourteen different groups of experiments are shown in Figure 4-5. Averaging the estimates of \( \alpha \) and \( k \) across all fourteen groups of experiments gives \( \alpha = 0.03 \pm 0.02\text{mm}^2/\text{s} \), and \( k = 0.002 \pm 0.002/\text{s} \), where the variability is given by the sample standard deviation. Visual inspection of the results for the thermal diffusivity (Figure 4-5A) indicates that the majority of estimates of \( \alpha \) for each experimental group lie within one standard deviation about the mean. Since there appears to be no discernible trend in the mean estimates of \( \alpha \) for varying burn duration, burn temperature and skin thickness, it is reasonable to characterize \( \alpha \) using a constant for all experimental scenarios considered.
Results for the loss rate (Figure 4-5B) indicate that the variability between estimates of $k$ between different groups of experiments is greater than the variability of $\alpha$ between different groups of experiments. Our results suggest that the thermal loss plays an insignificant role in short duration burn conditions, whereas our estimates of $k$ are larger for the longer duration burn conditions. It is difficult to speculate about the reason for this, however it is possible that for longer duration exposures ($\geq 300$ seconds), there is an increase in the rate of heat loss to the blood supply. In response to heat exposure, vessels in the local tissue area may dilate, increasing blood perfusion and enhancing heat dissipation. Increased perfusion to the local tissue and surrounding tissue may take several minutes to reach maximal effect.

Our estimates of $\alpha$ allow us to predict the approximate amount of time required for the thermal energy to propagate vertically down, through the skin, as $H^2/\alpha$. For the 12 week old animals, with $\alpha = 0.03 \text{ mm}^2/\text{s}$ and $H=2.27\text{ mm}$, the time taken will be approximately 170 seconds. Whereas, for the 5 week old animals, with $H=1.40\text{ mm}$, the time taken will be approximately 70 seconds.

Our average estimate of $\alpha$ is approximately 3–10 times smaller than previously reported *in vivo* estimates from 1947 (194). There are several possible explanations for this difference. A key difference between our experiments and those of Henriques and Moritz (194) is that we directly measure the transfer of thermal energy vertically down, through a layer of skin. In contrast, Henriques and Moritz measure the caloric uptake of skin, and then convert this quantity into an estimate of thermal diffusivity by making assumptions about the density and thermal conductivity of the skin. Another important difference is in the experimental design. Henriques and Moritz (194) create a burn using a heated copper disk, whereas we create scald burns using hot water. Furthermore, advances in technology allow us to use modern, calibrated and reproducible measurement instrumentation, whereas the previous results from 1947 relied on custom-made measuring instruments.
Figure 4-5 Boxplots showing estimates of $\alpha$ and $k$ for all burn conditions. The boxplots are created with MATLAB (219). The solid black horizontal lines indicate the sample mean across all experimental burn conditions, and the grey shaded region indicates the mean ± one sample standard deviation to illustrate the variability across all experimental conditions. Results in A. show estimates of the thermal diffusivity, the sample mean is $\alpha = 0.03 \text{ mm}^2/\text{s}$ and the sample standard deviation is $0.02 \text{ mm}^2/\text{s}$. Results in B. show estimates of the loss rate, the sample mean is $k = 0.002/\text{s}$ and the sample standard deviation is $0.002/\text{s}$.
4.6 Conclusions

In this work we consider a series of scald burns in an \textit{in vivo} porcine model. We choose to work with a porcine model because pig skin is anatomically and physiologically similar to human skin (65, 69, 70). Our experimental design allows for the collection of high quality \textit{in vivo} data, with multiple replicates performed on different animals, and at different anatomical locations for the same burn condition. Here, we examine how the thermal properties of skin depend on burn duration, burn temperature, and skin thickness. It is important to consider these variables because thermal injuries may occur under many different conditions. To the best of our knowledge, the experimental data set presented here is the first \textit{in vivo} study to directly compare heat conduction in thin and thick skin (representative of a human child and adult skin) subject to the same burn conditions.

To provide additional quantitative insight into our experimental data set we also interpret our data with a mathematical model. In particular, the focus of using the mathematical model is to provide estimates of the thermal diffusivity and the thermal loss rate, and to explore whether these estimates depend on burn duration, burn temperature or skin thickness. Our overall estimates are $\alpha = 0.03\text{mm}^2/\text{s}$ and $k = 0.002/\text{s}$. For long duration, moderate temperature burns, our estimates of $\alpha$ appear to be independent of the burn duration, burn temperature and skin thickness. In contrast, our estimates of $k$ are more variable. The model calibration procedure suggests that there is virtually no thermal loss during the very short time burn duration experiments whereas a larger loss rate is relevant for the longer duration burn conditions.

Both experiments and modelling suggest avenues for future research. Future experiments may include multiple temperature measurements at different depths, which would allow for a greater understanding of how heat is transferred through the different layers of the skin. In particular, whether heat transfer may change at different dermal depths and potentially affect the predicted time required to create a deep dermal versus a superficial dermal burn. Additionally, placement of the temperature probe using ultrasound guidance could be used, which would also enable \textit{in vivo} quantification of probe depth. Further investigations regarding the thermal loss rate and the role of blood perfusion could also be studied by repeating experiments on skin with no blood perfusion (non-living tissue). Extensions of our modelling framework could include spatial effects, but this would require additional experimental data.
Our estimates of $\alpha$ can be applied with most confidence to inform modelling studies of heat transfer where skin is exposed to moderate temperatures for relatively long durations, similar to the experimental procedure presented here. For example, this could include burn prevention strategies (78, 95, 109), predicting thermal damage for accidents (220), forensic medicine (215, 216) and thermal therapy studies (221). Importantly, improving understanding of temperature conduction in the skin is a vital first step towards examining the relationship between heat conduction and tissue damage, which has significant clinical applications. Furthermore, all data used in our mathematical model calibration procedure is available for re-use in future studies (Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.ijheatmasstransfer.2016.05.070.). This could be useful to enhance experimental design and to minimise animal use.

The data and modelling presented in this paper are part of a broader study investigating the pathophysiology of burns. The mathematical modelling completed on this simplified dataset enables an initial examination of the complex process of heat conduction in living skin and further work examining the relationship between heat conduction and tissue damage is currently underway. Overall, our study highlights how collaboration between researchers with expertise in animal/laboratory based experimental studies and theoreticians who specialise in applying mathematical models to interpret biological or clinical data can enrich the overall value of results obtained.

4.7 Acknowledgements

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4.8 Chapter Conclusion

This chapter addressed one of the secondary aims of this thesis which was to improve understanding of heat conduction through the skin. Findings from this study have provided valuable, quantitative information about the thermal properties of living skin, improving upon previous estimates obtained by extrapolating from *ex vivo* experiments.

This data has broad application to future heat transfer modelling investigations, such as those used to develop thermal injury prevention and treatment guidelines. For example, estimates for the thermal diffusivity and modelling established in this chapter have recently been used to inform a mathematical model examining changes in the temperature profile of the skin with the application of water as a first aid for burns (222). Importantly, while beyond the scope of this research project, the modelling presented in this chapter is vital for studies in this area investigating the more complex relationship between heat conduction and the severity of tissue damage.

Heat diffusivity is one variable that will affect heat damage and its measurement. Another factor which affects damage measurements is skin thickness and how that is assessed. The chapter that follows describes a study evaluating changes in skin thickness measurements with excision and biopsy processing procedures.
Chapter 5  
Optimisation of skin thickness measurements

This chapter is based on a publication in the journal Wound Repair and Regeneration.

Citation:

5.1 Chapter foreword

It is important to consider how variations in the thickness of the skin with age (and/or anatomical location) may alter the susceptibility to thermal insult. At the commencement of this research project, juvenile (3–4 month old) pigs were considered ideal to model adult human skin, having a similar skin thickness of 2–2.3mm (43, 94). Initially, testing for a broad range of burn conditions in the younger (5 week old) pigs, whose skin thickness was thought to more closely approximate that of a young child (1–1.3mm) (94), was proposed. However, part way through this research project, use of an ultrasound machine became available allowing in vivo measurement of skin thickness. Subsequently, substantial discrepancies between in vivo and ex vivo skin thickness measurements were observed. The following chapter describes how these discrepancies between in vivo and ex vivo measurement techniques were further evaluated. Findings from this study altered the planned progression of this research project, the impact of which is discussed in the chapter conclusion.
Skin thickness measurements increase with excision and biopsy processing procedures

5.2 Introduction

For researchers and pathologists examining skin samples, comparisons between in vivo measurements and histologically determined values for skin thickness are often required. Recently, during development of a porcine burn model (223), we observed skin thickness measured histologically was substantially thicker than in vivo ultrasound measurements. Anecdotally a generalised ‘shrinkage’ of skin sample dimensions with formalin fixation is well accepted, with reports of decreasing thickness post-processing for excised human skin tumours (99) and porcine and human cadaver skin (224). However, similar to our observations, others report increased thickness for human (100), dog (225) and cat (226) skin post-processing. Therefore, we designed a study to determine the magnitude of change and the influence of excision and biopsy processing procedures on porcine skin thickness measurements. This study compares in vivo ultrasound-derived thickness measured on living animals to three different histological processing techniques. Importantly, to our knowledge, this is the first study to investigate how pinning of a skin sample to approximate in vivo size and tension during processing affects skin thickness measurements.

5.3 Methods

Ethics approval for six female Large White pigs (35kg, 15 weeks of age) was obtained as part of another study (University of Queensland, QCMRI/446/15/QCHF). Pigs underwent a general anaesthetic, hair was clipped and the 3cm x 2cm sampling site marked (Figure 5-1A) in cranial and caudal positions on the lateral thorax (4 samples/pig, Figure 5-1D). Skin ultrasound was performed in vivo (Figure 5-1E), then ex vivo immediately after euthanasia and scalpel excision (Figure 5-1B, F) ((22MHz hockey stick probe (LOGIQ e R7 series, GE Healthcare) in B-scan mode)). Ultrasound measurements for dermal and total skin (epidermis + dermis) thickness were assessed at three points in a cross-sectional image. Length and width of excised skin was calculated from digital photos (Figure 5-1C) using Aperio ImageScope (Leica Biosystems, Wetzlar, Germany).
Figure 5-1 **Comparison between sampling and measurement techniques for one replicate.** Images of a skin sample (3cm x 2cm, *in vivo*) A. prior to excision, B. post-excision in situ and C. bench top. Schematic drawing of skin sampling locations D. on the cranial [1] and caudal [2] aspect of the lateral thorax. Ultrasound images E. *in vivo* and F. *ex vivo*. Haematoxylin and Eosin stained sections at 2X magnification for G. standard formalin, H. pinned formalin and I. frozen.

Using pre-drawn 1cm x 1cm (*in vivo*) lines as a template, each sample was divided and processed for histology: 1) 10% formalin for 24-36hrs, 2) skin edges stretched to *in vivo* dimensions (1cm x 1cm) and pinned to cork then placed into 10% formalin for 24-36hrs, and 3) snap frozen in Cryo-Embedding Compound (Pelco®), then stored at -80°C. Images of Haematoxylin and Eosin stained 5µm thick paraffin sections were captured with a Nikon EP600 microscope (Nikon Instruments Inc, USA) fitted with a Spot RT slider cooled CCD camera (SPOT Imaging Solutions™, Sterling Heights, USA). Dermal and total skin thicknesses were calculated using Image Pro Plus v.5.1 software (Media Cybernetics, Silver Spring, USA). Comparison between measurement techniques was analysed using a one way analysis of variance (ANOVA) with Tukey and Dunnet multiple comparison tests (alpha 0.05, 95% CI of difference) in GraphPad Prism V7 (GraphPad Software, Inc. California, USA).
5.4 Results and Discussion

The dermis has a high content of elastin and excision reduces the normal skin tension, allowing the dermis to recoil. Mean length and width shrinkage after excision was 12% and 19% respectively, similar to shrinkage values reported elsewhere for excised human skin tumours; 16% and 18% (101); 17% and 9% (227). As collagen and elastin fibres are largely orientated parallel to the skin surface, retraction is presumably greater horizontally leading to an overall thickening of the sample vertically. However, *ex vivo* ultrasound measurements were only 5% thicker than *in vivo*. While this compares to the 6% observed after excision of human skin tumours (99), it is substantially less than the 46% increase reported for 4mm biopsies (100). This disparity may be explained by differences in biopsy size and scanning techniques between the studies.

The dermis and total skin were significantly thicker (*p* < 0.0001) after histological analysis of routinely processed formalin and frozen sections than *in vivo* ultrasound measurement (Figure 5-2A, B). The mean relative thickening was 54% (dermis) and 41% (total skin) for formalin-fixed, and 66% (dermis) and 51% (total skin) for frozen-fixed sections. Tan *et al.* reported a larger increase for total skin thickness of 76% (formalin) and 88% (frozen), however, they analysed smaller samples (4mm) of human skin (100). We have observed (data not shown) a relative thickening of approximately 73% for 8mm punch biopsy samples. The size, shape and method of sample collection likely contributes to discrepancies between studies and the magnitude of effect may increase as the excised sample size decreases. Our results are similar to studies comparing histology to *ex vivo* measurement reporting an increased thickness of 55% for cat skin (1.2 x 6cm ellipse) (226) and 65% for dog skin (8 x 4cm ellipse) (225). Similar to Tan *et al.* (100), frozen-fixed sections were thicker than formalin-fixed, although this was not statistically significant.
Figure 5-2 Comparing skin thickness measurements of the five different methods. For A. dermis and B. total skin (epidermis + dermis), * significantly different from ultrasound in vivo (p < 0.0001).

Pinned formalin sections were significantly thinner than those processed via standard formalin (p < 0.0001). Pinned sections had a more compact appearance and the epidermis was flattened with less prominent ridging (Figure 5-1G, H). Grossly, pinned samples had a dome like shape due to tautness at the pinned corners and overstretching of the epidermis may have contributed to thinner than anticipated total skin thickness values for this group. However, pinning the skin sample to approximate in vivo size and tension during processing resulted in values most comparable to those obtained in vivo. Future avenues for research include refining the pinning technique and use of higher frequency ultrasound probes to improve resolution of the dermoepidermal junction, allowing the relative changes seen in the epidermis to be compared to the dermis.

Site specific variations in skin tension and or thickness may affect the magnitude of change seen post-processing. The cranial site near the forelimb has greater skin tension, a larger increase (not statistically significant) in the mean relative thickening was observed; 62% (dermis) and 46% (total skin); compared to the caudal site mean of 46% (dermis) and 37% (total skin) for formalin-fixed sections. This trend was similar for frozen-fixed sections. Additional site specific variations at different body locations were not investigated and are a limitation of this study. Furthermore, only normal skin was analysed and it is unknown how changes to the tissue architecture with disease or pathology may alter responses to processing. Additionally, while pig skin has a similar amount of collagen to humans, it has a lower elastic content (67), thus a greater change from in vivo dimensions could be anticipated for human skin.
5.5 Conclusion

In summary, a significant increase in thickness after excision and processing of 54% (dermis) and 41% (total skin) for formalin-fixed sections was observed. The significant disparity between *in vivo* and *ex vivo* thickness measurements should be considered when absolute and to a lesser degree relative values of depth are reported. In particular, overestimation of depth as a result of processing artefact is a likely outcome. To best approximate *in vivo* thickness for a large skin sample, pinning during formalin fixation is recommended. For smaller samples (e.g. punch biopsies) or frozen sections where pinning is not feasible, substantial artefactual thickening post-excision and processing is anticipated.
5.6 Chapter Conclusion

The study presented in this chapter confirmed initial observations that skin thickness measured *in vivo* with ultrasound was thinner than *ex vivo* determined with histology. Publishing these results increases awareness regarding the likelihood of substantial artefactual thickening of skin post-excision and processing. These findings have broad application to clinicians, pathologists and all researchers working with skin.

As explained in the chapter foreword, prior to procurement of an ultrasound which allowed for *in vivo* skin thickness measurements, it was anticipated that burn conditions would be investigated in younger weaner pigs (5 weeks old, 10kg). Previous to this study, based on histological measurements, it was assumed that 12 week old pigs had a skin thickness more similar to adult humans (2–2.5mm). However, ultrasound measurements obtained from the porcine studies (presented in chapters 3 and 6) indicated the *in vivo* skin thickness of a 12 week old pig is similar to that of a human child (1–1.4mm). This similarity in skin thickness is important as the motivation for this research project was to evaluate scald injury severity in children.

Although it would have been desirable to continue investigations with both the 12 and 5 week old animals, this was not ethically or logistically feasible for this project. Therefore, moving forward, it was determined that the main experimental animal studies for this thesis would continue to be conducted using only 12 week old animals. The next section of this thesis describes the main results and findings from these experimental animal studies.
Chapter 6  Water temperature and duration of exposure for deep dermal scalds

This chapter is based on a paper published in the journal Wound Repair and Regeneration.

Citation:

6.1 Chapter foreword

This chapter describes and discusses the main results and findings from the experimental animal studies forming the bulk of the work conducted during this research project. Evidence-based injury prediction data is presented for the burn conditions (water temperature and duration of exposure) required to sustain clinically relevant deep dermal scald injuries. The porcine scald model, developed and optimised in the preceding chapters, was used to test a broader range of burn conditions and the extent of tissue injury was quantified with histological analysis. Additionally, burn conditions demonstrating mid-to-deep dermal damage were further evaluated and the clinically relevant outcome of time to re-epithelialise was assessed. Important findings regarding the association between histologically determined burn severity (utilising vascular damage as a marker of tissue injury) and re-epithelialisation outcome are also reported here.
Evidence-based injury prediction data for the water temperature and duration of exposure for clinically relevant deep dermal scald injuries

6.2 Abstract

Deep dermal burn injuries require extensive medical care, however, the water temperatures and durations of exposure that result in a severe scald injury are unknown. This study used a porcine burn model to investigate the time and temperature threshold for clinically relevant deep dermal injuries for both immersion (long duration) and spill/splash (short duration) scald events. Scald wounds were created on the flanks of anaesthetised juvenile Large White pigs (27kgs). Acute tissue injury evaluations performed at 1 hour and days 1, 3 and 7 post-burn (16 pigs) included: wound examination, biopsies and Laser Doppler Imaging. Up to 20 burn combinations were tested including: 50–60°C water for 1–10 minutes (immersion); and 60–90°C water for 5 seconds (spill/splash). Burn conditions demonstrating mid-to-deep dermal damage histologically were followed for 21 days to assess time to re-epithelialise (8 pigs). Histologically, depth of damage increased until day 3 post-burn. Damage to ≥ 75% of the depth of dermis was associated with burns taking longer than 3 weeks to fully re-epithelialise. For spill/splash (5 second) scalds, water at ≥ 75°C showed damage to mid-dermis or deeper by day 3, however, only burns from water ≥ 85°C were not fully re-epithelialised by day 21. For immersion scalds of equivalent duration, water at 55°C caused significantly deeper dermal damage than 50°C (p < 0.05) at day 3. Immersion scalds which were not fully re-epithelialised by day 21 included 50°C for > 10 minutes, 55°C for 5 minutes, 60°C for 60 seconds, and 70°C for > 15 seconds. This research provides valuable evidence-based injury prediction data, which can be used to inform future burn injury prevention guidelines/legislation to reduce the risk of severe scald injuries and support medicolegal opinions for cases where an inflicted mechanism of injury is alleged.

6.3 Introduction

Burns are a global public health problem and are particularly common in early childhood (228). Scalds are the most commonly treated burn injury in young children (10). To reduce the risk of scalding, understanding the relationship between water temperatures, duration of exposure and tissue injury depth and/or severity is essential. Scald injuries may result from exposure to very hot liquids for only a short duration i.e. ‘spill/splash’, and also as a result of ‘immersion’ in more moderate temperature liquids for relatively longer durations. While all
burn injuries are painful and distressing for a child, severe burns require hospitalisation for extensive medical treatment, surgical intervention and in the long term often result in scarring and disfigurement. Therefore, it is essential to investigate not only the burn conditions predicted to result in cutaneous injury, but also to consider the likely severity of injury, with particular reference to the more serious and clinically relevant deep dermal and full thickness burns.

In both clinical and research settings, the importance of standardised burn injury severity classification is well recognised (31, 79). Clinically, timely and reliable identification of burn injury severity is essential to guide management and treatment decisions. Of particular importance is distinguishing between; superficial partial thickness (SPT) burns which are predicted to fully re-epithelialise within two to three weeks and are unlikely to scar; and more severe deep dermal partial thickness (DDPT) burns which take longer than 3 weeks to fully re-epithelialise, require surgical intervention and result in scarring (2-8). However, accurate and timely clinical assessment of burn depth can be challenging even for experienced clinicians (105, 116, 117). Therefore, for cases where detailed information regarding the circumstances of the scald injury event are available, access to updated severity of injury prediction data would be a useful adjunct to inform treatment and decisions regarding referral of patients to burn centres. Additionally, for clinicians providing ‘expert’ medicolegal opinion for cases where an inflicted scald injury mechanism is suspected, there is currently no valid evidence-based injury prediction data available to consider the burn conditions expected to cause a severe deep scald injury.

International approaches to hot water safety are predominantly based on scald injury prediction data from the seminal work of Moritz and Henriques published in the 1940s (1, 33, 229). Using a porcine model, Moritz and Henriques investigated the time and temperature threshold at which scald burns occurred and showed that the duration of exposure to cause a partial thickness burn reduced as the water temperature increased. Continuing this idea, they conducted limited experiments on human subjects establishing threshold exposures for the shortest duration of a given water temperature to result in cutaneous injury (irreversible epidermal damage). However, a key limitation of the Moritz and Henriques study is that only one microscopic or pathological indicator of thermal injury was reported; whether or not the epidermis was completely destroyed. As a result, data from Moritz and Henriques thermal studies have been and continue to be erroneously translated and extrapolated such that full thickness damage to the epidermis is incorrectly equated to
a more severe full thickness burn (48, 49). Additional limitations in the methodological approach taken by Moritz and Henriques (1) further contribute to uncertainties regarding the validity of their threshold results for ‘deep dermal’ damage. For example, microscopic evaluation of human burns was not performed; detailed quantitative histological results for depth of injury to the dermis were not presented; the number of replicates of each burn condition was not standardised (many burn conditions were not replicated); and the ultimate fate of the burns, determined by time to re-epithelialise, was not reported. Despite the international importance of this area of research for scald prevention, evidence-based injury prediction data for the burn conditions to cause deep rather than superficial dermal injuries is lacking.

Pigs are accepted as an appropriate animal model for thermal injury investigations given the physiological and anatomical similarities between human skin and pig skin (44, 67, 69, 70). For this study, a previously developed porcine scald model (223) was used. The primary aim of this study was to establish the burn conditions (water temperature and duration of exposure) to cause clinically relevant severe spill/splash or immersion scald injuries. The two main outcome measures used to assess burn injury severity were histologically determined depth of tissue injury and time to wound re-epithelialisation (healing). Secondarily, the association between histologically determined burn severity (using vascular damage as the predominant marker of tissue injury) and re-epithelialisation outcome was investigated and the threshold value (percentage of the total dermal thickness damaged) which resulted in a severe deep dermal injury was determined.

6.4 Materials and methods

6.4.1 Ethics Statement

All methods conformed to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes (7th Edition) published by the Australian National Health and Medical Research Council. Ethics approval was obtained from the University of Queensland Animal Ethics Committee (Approval numbers: QCMRI/RCH/326/12/QCMRI/NHMRC and QCMRI/446/15/QCHF). All procedures were performed under a general anaesthetic and all animals were treated in a humane manner.

6.4.2 Animals

Juvenile Large White (female) pigs of approximately 12 weeks of age (27kg) were used for
the study. To allow for acclimatisation, animals were delivered to the animal house 7 days prior to commencing experiments. Animals were given free access to water and fed a standard grower feed pellet diet. Animals were fasted overnight on days prior to procedures requiring administration of anaesthesia. Scald burns were created on the pigs whilst they were under a general anaesthetic.

The method of general anaesthesia and monitoring was exactly as described by Andrews et al. (223). Briefly, anaesthesia was induced with Ketamine 13mg/kg (Ketamine 100mg/ml Ceva™, Glenorie, NSW, Australia) and 2mg/kg Xylazine (Ilium Xylazil 100mg/ml, Ilium™, Troy laboratories Pty Ltd, NSW, Australia) and maintained with 1–2.5% Isoflurane (Attane, Bayer Australia Ltd, Pymble, NSW, Australia). Buprenorphine 0.01mg/kg (twice daily, Temgesic® 0.3mg/ml Reckitt Benckiser Healthcare, Slough, Berkshire, UK) and Fentanyl 50µg/hr patches (every 3 days, Durogesic®50, Janssen-Cilag Pty Ltd, North Ryde, Australia) were administered as needed to provide analgesia. Animals underwent the same general anaesthetic regime at each dressing change and/or biopsy collection time point. At the end point of each experiment, animals were euthanased with sodium pentobarbitone (Lethabarb™, Virbac Pty Ltd, NSW, Australia).

6.4.3 Burn Model and Experimental Protocol

Circular scald wounds, approximately 16cm², were created using a purpose made scald device (223). Pre-heated water (set at a desired test temperature) was pumped into the device and vacuum suctioned out at an equivalent rate, ensuring the water temperature was constant for the required exposure time. Burns were located on the lateral aspect of the thorax, with four burns each side, giving a total of eight burns per pig. A comprehensive description of this model including location of burns, description of the device and temperature monitoring was previously reported by Andrews et al. (223).

The extent of tissue injury in the acute post-burn period was investigated using 16 pigs. Visual wound examination, full thickness 8mm punch biopsies and Laser Doppler Imaging were performed in conjunction with dressing changes at 1 and 24 hours and at 3 and 7 days post-burn. In total, 20 burn combinations were tested including 50–60°C water for 1–10 minutes (representative of a long duration hot tap water immersion injury) and 60–90°C water for 5 seconds (representative of a short duration spill/splash scald injury). Each burn combination had a within-animal replicate as well as biological replicates (at least 2 other animals). Subdermal temperature probes (K type thermocouples, Radiospares Components
Pty LTD., Smithfield, Australia) were used to monitor the temperature within the skin during scald creation; results for subdermal temperature change have previously been reported by our group (189, 223) and are not presented here.

Using 8 pigs, burn conditions from the acute study which demonstrated mid-to-deep dermal damage (determined by histological examination at day 3) were then followed for 21 days in a separate experiment to assess the clinically relevant outcome of time to re-epithelialise. The same method was used to create and evaluate burns as the first experiment with the following exceptions to minimise disruption to healing: wound assessments and dressing changes were performed at days 3, 7, 10, 14, 17 and 21 post-burn; biopsies were only taken at day 21 (end point of experiment); and Laser Doppler Imaging (LDI) was performed at days 3 and 7 post-burn. A total of 10 burn combinations were tested including 50°C for 10 minutes, 55°C for 2 and 5 minutes, 60°C for 30 seconds and 1 minute, 70°C for 15 seconds, and 75°C, 80°C, 85°C and 90°C for 5 seconds.

To measure in vivo skin thickness, ultrasound was performed prior to scalding using a 22MHz hockey stick probe (LOGIQ e R7 series, GE Healthcare, Parramatta, NSW, Australia) in B-scan mode. Values for mean dermal and total skin thickness (epidermis + dermis) were obtained from measurement at three points in a cross-sectional image.

At dressing changes, burns were cleaned to remove exudate and dried eschar with sterile swabs soaked in 0.1% chlorhexidine gluconate solution (Hospital Pharmacy, Brisbane). All burns received no other treatment. After wound assessments, burns were dressed with Mepilex® (Möllycke Health Care, Gothenburg, Sweden) and Fixomull® (Smith & Nephew, North Ryde, NSW, Australia). Custom made garments (2) were fitted to further protect the burn area.

6.4.4 Wound Assessment

6.4.4.1 Clinical observation

At each time point, wounds were examined visually and digital photographs were taken using a Canon EOS 300D digital SLR camera (Canon Australia, North Ryde, Sydney). The total area of each burn (cm²) was assessed using a Visitrak™ device (Smith & Nephew, Australia) (2, 223). Wound re-epithelialisation was assessed using a previously described method (157) where the Visitrak™ device was used to calculate the amount of wound re-epithelialisation as a percentage of total wound area by tracing the outlines of moist,
unhealed skin areas (un-re-epithelialised skin). For this study, wounds ≥ 95% re-epithelialised were considered fully re-epithelialised.

6.4.4.2 Histological investigation

6.4.4.2.1 Depth of injury determined histologically

For the acute post-burn histological experiment, biopsies were obtained from all burns and normal skin at 1 hour, and days 1, 3 and 7 post-burn. The circular burn area was divided into quadrants and sequential biopsies were taken from the centre of each quadrant at different time points, resulting in individual burns having a total of 4 well-spaced biopsy sites by day 7 post-burn. All histopathologic evaluations were performed on formalin-fixed (10% neutral buffered formalin for 24 hours), alcohol-dehydrated, xylene-cleared, paraffin-embedded, Haematoxylin and Eosin (H&E) stained 5 µm thick sections. Sections were digitally captured using a Nikon EP600 microscope (Nikon Instruments Inc, USA) fitted with a Spot RT slider cooled CCD camera (SPOT Imaging Solutions™, Sterling Heights, USA) and scored by an examiner blinded to sample details other than time post-burn. To exclude any batch effect, the complete data set was measured at the conclusion of all testing.

Using Image Pro Plus v.5.1 software (Media Cybernetics, Silver Spring, USA) quantitative measurements (in mm) for the minimum, maximum and average thickness of the dermis and depth of dermal damage over the entire section were calculated electronically (rather than manually averaging measurements taken from a few selected points). The histological markers of tissue injury observed in H&E sections has been previously described by our group (223) and include: blocked vessels, endothelial cell injury, adnexal necrosis, infiltration of inflammatory cells, and dilation of lymphatic vessels. In agreement with Singer et al. (55), the line of damage was initially observed to be consistently deeper for vascular injury (including vascular occlusion, extravasation of erythrocytes and endothelial cell necrosis) compared to adnexal necrosis or collagen injury. Therefore, while all dermal elements were evaluated for damage, the line of damage represents the deepest level of vascular injury detected for each section. The average depth of this line was used to calculate results presented here as % injury to dermis by depth (average depth of damage to dermis divided by the average total thickness of the dermis).

6.4.4.2.2 Histological analysis of re-epithelialisation

For the time to re-epithelialise investigations, at day 21 a representative 1 x 1cm full thickness biopsy was excised from the centre of the burn injury. Re-epithelialisation
percentage was calculated from 5µm H&E stained sections (one field of view at 2 times magnification) by measuring the length of the neoepidermis and dividing this by the total length of the section.

6.4.4.3 Laser Doppler Imaging

To assess blood flow to the wound, Laser Doppler Imaging (Moor LDI 2, Moor Instruments, Devon, UK) was performed at 1 and 24 hours for acute post-burn studies and days 3 and 7 for all burns. The method of scanning and image analysis was as previously described (223). Briefly, a large scanning area with high resolution (256 X 256 pixels) and a slow scan speed of 4 ms/pixel was used. By defining each individual burn area as a “region of interest” (ROI), the average perfusion units (PU) per wound were calculated. To standardise comparisons between different time points for the same animal and between animals, burn perfusion levels relative to normal perilesional skin are presented rather than absolute perfusion units. For each flank, one perilesional ROI of normal skin was measured as a control. Results for LDI imaging are given as the mean blood perfusion ratio of burned skin (B) to normal (N) perilesional skin. A wound with higher than normal blood flow (B/N > 1) is representative of a more superficial injury and lower flow than normal (B/N < 1) indicates a full thickness injury. However, a blood flow similar to normal (B/N = 1) can also be seen with a deep dermal injury.

6.4.5 Data analysis

GraphPad Prism V6 (GraphPad Software Inc., San Diego, California, USA) and SPSS 22 (IBM Corporation, Armonk, N.Y, USA) were used to perform statistical analysis. Histological depth of damage was analysed using Univariate Analysis of Variance for between subject effects and pairwise comparisons. A binary logistic regression model was used to analyse re-epithelialisation data, where the reference category was burns which had fully re-epithelialised (≥ 95% re-epithelialised) by day 21. Results are reported as mean ± standard error of the mean (SEM) unless otherwise stated.

6.5 Results

In total, 24 pigs were used for the study. One pig had an adverse anaesthetic reaction and was euthanized at 24 hours post-burn, all burns from this pig were excluded from analysis. In total 115 individual burns were included for histological analysis in the early post-burn period and a further 61 burns were followed to re-epithelialisation (21 days). The average
Burn size was $17.5 \pm 0.7\text{cm}^2$, with a length of $4.9 \pm 0.1\text{cm}$ and a width of $4.7 \pm 0.1\text{cm}$. The average weight of the pigs was $26.6 \pm 3.6\text{kg}$. The total skin thickness (epidermis and dermis) \textit{in vivo} was $1.42 \pm 0.23\text{mm}$ (measured using ultrasound) and \textit{ex vivo} $2.09 \pm 0.20\text{mm}$ (determined histologically).

6.5.1 Burn conditions resulting in moderate severity injury (mid-to-deep dermal damage) can be assessed histologically

The extent of damage to the dermis determined by histological assessment at 1 hour and days 1, 3 and 7 post-burn for each burn condition is shown in Figure 6-1. Mid-dermal injury was defined as damage extending between 25% and 75% of the depth of the dermis and was considered a moderate severity injury.

![Figure 6-1](image)

Figure 6-1 Relative injury to the dermis determined by histological assessment at 1 hour and days 1, 3 and 7 post-burn for each condition. A. Spill/splash scalds (5 second duration); immersion scalds for water at B. 50°C, C. 55°C and D. 60°C. The dotted black line shows level of 25% and 75% depth of dermis damaged, burns between these lines were considered moderate severity. Results expressed as average ± SEM, $n = 6$. 

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6.5.1.1 For spill/splash scalds (5 second duration), water temperatures in excess of 75ºC were associated with mid-to-deep dermal damage

For the majority of spill/splash scalds, the depth of damage continued to increase until day 3 post-burn (Figure 6-1A), where the deepest depth of damage was observed. At day 3, there was a significant association between increasing water temperature and increasing severity of damage observed as % depth of dermis damaged (p = 0.002). Scalds ≤ 70ºC had substantially less dermal damage than scalds 75ºC or higher, however, this was only statistically significant for the 90ºC scalds (p < 0.05). By day 3, water at ≥ 75ºC showed evidence of damage to the level of the mid-dermis or deeper. At day 7, water at 90ºC caused significantly (p < 0.05) greater dermal damage than water 60-85ºC.

6.5.1.2 For immersion scalds, increasing the duration of exposure results in significantly increased depth of dermal damage

For the lowest intensity burn conditions tested (50ºC for 1–5 minutes, 55ºC for 1 minute) the deepest depth of damage observed was at 1 hour post-burn (Figure 6-1B, C). For these burns much of the initial vascular congestion seen at 1 hour post-burn was not apparent at 24 hours, indicating this congestion was transient and not representative of irreversible damage to vessels. In general, for more moderate to severe immersion scalds, the depth of damage to the dermis was deepest at 24 hours post-burn and not observed to increase substantially at subsequent time points.

At day 3 post-burn a significant relationship between increasing duration of exposure and increasing depth of dermal damage was observed for 50, 55 and 60ºC water scalds (p ≤ 0.005). For equivalent durations of exposure, water at 55ºC caused significantly deeper dermal damage than 50ºC (p < 0.05). The only 50ºC scald where tissue damage was observed to extend beyond the superficial dermis was the 10 minute exposure (60% depth of dermis damaged). For 55ºC water, exposure for 2 minutes resulted in damage to the level of mid-dermis (71% depth of dermis damaged), however, exposure for 5 minutes caused damage to the deep dermis (88% depth of dermis damaged). For 60ºC water, at day 3, damage to the level of the mid-dermis was evident after 30 seconds exposure (55% depth of dermis damaged), with damage observed to extend throughout almost the entire dermis with 1 minute exposure (98% depth of dermis damaged).
6.5.2 Burn conditions resulting in a clinically relevant deep burn which takes longer than 3 weeks to fully re-epithelialise

To minimise animal use, only burn conditions identified as moderate severity (mid-to-deep dermal damage) or deeper by histological evaluation at day 3 were used for time to re-epithelialise investigations. These conditions included 75°C, 80°C, 85°C and 90°C for 5 seconds, 50°C for 10 minutes, 55°C for 2–5 minutes, and 60°C for 30 seconds and 1 minute. In addition, 70°C for 15 seconds was tested as water at this temperature has been implicated in clinical reports of domestic hot water tap scald injuries (15 seconds was considered likely to result in a deep burn). The burns for which re-epithelialisation rates were observed included 24 individual spill/splash scalds (n = 6, four burn conditions) and 37 individual immersion scalds (n ≥ 6, six burn conditions). By day 21, many of the burns had completely re-epithelialised and were observed to have a mainly normal appearance with a just faint outline of the burned area. Other wounds which had not fully re-epithelialised were still moist and exudative. Burns which had not fully re-epithelialised by day 21 tended to have a red and white mottled appearance, with some areas of healed, normal, white skin and other unhealed areas with blood blisters and white eschar. Visual assessments of wound appearance and severity at 1 hour post-burn were not observed to reliably predict which burns would be ≥ 95% re-epithelialised by day 21 (Figure 6-2).
Figure 6-2 Appearance of wounds and re-epithelialisation observed until day 21 post-burn. A. Burns which had not fully re-epithelialised by day 21 (55°C/5min, 60°C/1min, 85°C/5sec, 90°C/5sec) had a red and white mottled appearance, with some areas of healed, normal, white skin and other unhealed areas with blood blisters and white eschar. Burns which had fully re-epithelialised by day 21 (50°C/10min, 55°C/2min, 60°C/30sec, 70°C/15sec, 75°C/5sec, 80°C/5sec) were observed to range in appearance from normal with only a faint outline of the burned area visible, to having patchy areas of pink, dry skin. The percentage of the burn which was re-epithelialised when assessed clinically and measured using a Vistrak™ device for B. spill/splash (5 second duration) and C. immersion scalds. The dotted black line indicates 95% re-epithelialised, burns above this line are considered to have full wound closure. Results are expressed as average ± SEM.

6.5.2.1 Assessment of re-epithelialisation rates for different conditions up to 21 days post-burn

By 21 days post-burn, 40 of the burns had fully re-epithelialised (≥ 95% re-epithelialised) and 21 of the burns had not. There was no statistically significant relationship between re-epithelialisation outcome for initial weight of the pigs, side of the burns, or site of the burns.

For spill/splash (5 second duration) scalds, an increase in water temperature was associated with a significantly (p = 0.03) worse re-epithelialisation outcome at day 21 post-burn. A binary logistic regression model was used to determine the probability of wounds being fully
re-epithelialised versus the probability of not fully re-epithelialised, and for every 1°C increase in water temperature, the burn was 1.149 times less likely to fully re-epithelialise (95% CI 1.012–1.304). For spill/splash scalds, a water temperature ≥ 85°C was observed to result in a burn which had not fully re-epithelialised by 21 days (Figure 6-2B). For immersion scalds, an increase in duration of exposure was associated with a significantly worse re-epithelialisation outcome for 55°C (p = 0.05) and 60°C (p = 0.009) water. The only immersion scalds which had not fully re-epithelialised by day 21 were 60°C for 1 minute and 55°C for 5 minute scalds (Figure 6-2C). There was excellent agreement between gross clinical evaluation and histologically determined re-epithelialisation at day 21 post-burn (results not shown). The burn conditions resulting in a clinically relevant deep burn (DDPT to full thickness (FT)) which takes longer than 3 weeks to fully re-epithelialise are summarised in Table 6-1.

Table 6-1 Temperature and duration of exposure for a clinically relevant severe scald injury which will not fully re-epithelialise within 21 days.

<table>
<thead>
<tr>
<th>Water Temperature</th>
<th>Duration of exposure</th>
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<tbody>
<tr>
<td>50°C</td>
<td>&gt; 10 minutes</td>
</tr>
<tr>
<td>55°C</td>
<td>2 ≥ 5 minutes</td>
</tr>
<tr>
<td>60°C</td>
<td>30 ≥ 60 seconds</td>
</tr>
<tr>
<td>70°C</td>
<td>&gt; 15 seconds</td>
</tr>
<tr>
<td>85°C and 90°C</td>
<td>5 seconds</td>
</tr>
</tbody>
</table>

6.5.2.2 Burns showing damage to ≥ 75% depth of dermis (determined histologically) are unlikely to fully re-epithelialise by day 21

Direct comparison between histologically determined burn severity and the re-epithelialisation outcome for an individual burn was not possible as burn wounds studied for re-epithelialisation did not have biopsies taken (which can disrupt healing). Therefore, the association between histologically determined burn severity and re-epithelialisation outcome was analysed by considering the relationship between the average % depth of the dermis damaged determined histologically at day 3 and re-epithelialisation at day 21 (assessed clinically) for each burn condition (Figure 6-3A). As a result of this analysis, groups of wounds were identified post-hoc: those which had fully re-epithelialised by day 21 had < 75% depth of dermal damage at day 3; those which had not fully re-epithelialised by day 21 showed deeper damage at day 3, with ≥ 75% depth of dermis damaged (except 85°C for 5
seconds).

The time and temperature relationship for a DDPT scald burn which will not fully re-epithelialise by 21 days compared to threshold values for dermal injury (determined histologically) of ≥ 50% or ≥ 75% of the dermis damaged is shown in Figure 6-3B. Classifying a clinically relevant DDPT burn using a threshold value of 50% depth of the dermis damaged overestimated the burn severity as many of these burns were seen to fully re-epithelialise by 3 weeks post-burn. Additionally, the time and temperature relationship for a deep scald burn reported by Moritz and Henriques in the 1940s (1) is presented and compared to data from this study (Figure 6-3B) and demonstrates how their results also overestimate injury severity. However, when the time and temperature threshold line was defined as ≥ 75% of the dermis damaged, this gave an excellent indicator of the clinical severity of the burn wound, observed to almost directly overlay the data for burns which had not fully re-epithelialised by day 21.
The time and temperature threshold for a deep dermal partial thickness burn injury using different criteria to define tissue injury severity. A. Summarises results for histologically determined burn severity at day 3 post-burn and re-epithelialisation outcome at day 21. Mid-dermal 25% > 75% depth of dermis damaged; deep dermal ≥ 75% depth of dermis damaged. B. The time and temperature relationship for; damage to ≥ 50% of the dermis; damage to ≥ 75% of the dermis; and a DDPT scald burn which will not fully re-epithelialise by 21 days. The threshold line for ≥ 75% of the dermis damaged was observed to almost directly overlay the line for not fully re-epithelialised by day 21. Experimental data from the 1940s for 2º and 3º burns (identified by complete epidermal necrosis and a red or pale appearance) described by Moritz and Henriques (16) overestimates injury severity showing a threshold line most disparate from the line of not fully re-epithelialised by 21 days presented in this study.
Only burns showing evidence of extensive damage to vessels in the deeper portion of the dermis were associated with an injury which did not fully re-epithelialise by day 21. At 24 hours post-burn, for moderate severity burns (60°C for 30 seconds, 75–80°C for 5 seconds), the pattern of vascular damage to the mid-dermis (25% > 75% depth of dermis damage) was uneven within each section, with substantial intermingling of blocked and patent vessels observed (Figure 6-4B). However, for burns showing deeper dermal (≥ 75% of the dermis damage) to full thickness damage (55°C for 5–10 minutes, 60°C for 1–2 minutes) the pattern of vascular injury throughout the deeper dermis was more consistent and extensive (Figure 6-4C).

![Figure 6-4 H&E stained sections of normal (unburned) skin and burned skin at 24 hrs post-burn showing patterns of vascular injury. A. normal skin, thick black arrows identify hair follicles and glandular structures, predominantly located within the deep dermis. B. shows a mid-dermal burn injury (44% depth of dermis damaged) where a well-defined area of densely packed uniformly blocked vessels in the superficial dermis is shown above the black dashed line; and a more uneven pattern of vascular damage with increased intermingling of blocked (thin black arrows) and patent vessels (open white arrows) is evident in the mid-dermis. C. a full thickness burn injury showing more consistent and extensive vascular injury throughout the entire dermis.]

6.5.3 Laser Doppler Imaging

6.5.3.1 Repeat biopsies affect reliability of LDI scans

For the acute post-burn study, areas surrounding previous biopsy sites (from sampling at 1, 24 hours and 3 day post-burn) showed visible signs of disruption ranging from mild erythema to increased exudate and excoriation (Figure 6-5A). LDI scans from burns with previous biopsies showed areas of increased perfusion extending well beyond the margins of the original area biopsied (Figure 6-5A). While previous biopsy sites were excluded from calculations for average perfusion, increased perfusion in the region surrounding the biopsy sites was not excluded, as the size of this region was variable. This disturbance of the burn with repeat biopsies affected the reliability of LDI scans at days 3 and 7. For equivalent burn
conditions, consistently higher perfusion results were observed in burns which had previous biopsies compared to burns which did not (Figure 6-5B, C). The observed difference was greater at day 7 and was statistically significant for all spill/splash burns except 75°C for 5 second scalds. A similar trend was observed with immersion scalds, although this was only significant for 50°C for 10 minute and 60°C for 30 second scalds.

### 6.5.3.2 Repeat measurements improve the strength of LDI predictions regarding healing potential of moderate severity burns

Only LDI scans from burns without biopsies were included in further LDI analysis. The perfusion results for individual burn conditions are shown in Figure 6-5D, E. All burn conditions with a poor healing outcome (not fully re-epithelialised by day 21) showed lower perfusion than normal at day 3 (85°C for 5 seconds, 90°C for 5 seconds, 55°C for 5 minutes, 60°C for 1 minute). However, some burn conditions which fully re-epithelialised by day 21 also showed similarly low perfusion at day 3 (55°C for 2 minutes, 60°C for 30 seconds). Comparatively, at day 7 post-burn, a greater difference in perfusion between burn conditions for moderate and severe injury was observed, better predicting re-epithelialisation outcome. By day 7, burns fully re-epithelialised within 3 weeks had B/N ratios higher than 1 ((significant for 75°C for 5 seconds, 50°C for 10°C minutes and 70°C for 15 seconds (p = 0.02)).

The relationship between LDI scans at days 3 and 7 and re-epithelialisation outcome at day 21 was further analysed by grouping all burns into those which had or had not fully re-epithelialised for all burn conditions, as well as separately for spill/splash and immersion scalds (Figure 6-5F-H). Generally, burns with lower than normal perfusion (B/N ratio <1) at day 7 were associated with a poor re-epithelialisation outcome, however, this was not statistically significant with all burns included (p = 0.058, n = 61), or for immersion scalds (p = 0.267, n = 37). When only spill/splash scalds were included (n = 24), burns with lower B/N ratios at day 7 were associated with a significantly worse re-epithelialisation outcome at day 21 (p = 0.014). For spill/splash scalds, groups of fully re-epithelialised and not fully re-epithelialised burns had similarly low perfusion at day 3, but by day 7 the burns which went on to fully re-epithelialise by day 21 always had higher than normal perfusion, whereas low or similar to normal perfusion was observed for burns which had not fully re-epithelialised.
Figure 6-5 Results for Laser Doppler Imaging at days 3 and 7 post-burn. Biopsies from earlier time points affected reliability of subsequent LDI scans. A. For spill/splash (5 second duration) scalds at day 3 post-burn, the area surrounding previous biopsy sites shows signs of disruption ranging from mild erythema to increased exudate and excoriation. LDI scans of these burns show areas of increased perfusion extending beyond biopsy margins. Regions in grey indicate biopsy areas excluded from perfusion calculations. LDI B/N ratios comparing equivalent burns with and without biopsies for B. spill/splash and C. immersion scalds at days 3 and 7 post-burn. B/N perfusion for individual burn conditions for D. spill/splash and E. immersion scalds. Relationship between perfusion levels and re-epithelialisation outcome including F. all burn conditions and separately for G. spill/splash and H. immersion scalds. The dotted line (B/N ratio of 1) indicates normal perfusion. Burns < 95% re-epithelialised were considered not fully re-epithelialised. Mean ± SEM, n = 6, *p < 0.03, **p < 0.001.
For experienced burns clinicians, identifying and predicting the healing outcome of superficial severity burns and severe full thickness burns is less challenging than predicting the ultimate fate of more moderate severity injuries such as DDPT burns. This study aimed to establish evidence-based injury prediction data for the burn conditions (water temperature and duration of exposure) to cause a clinically relevant DDPT burn from a spill/splash or immersion scald injury event. Tissue injury severity was evaluated using visual wound assessment, histological analysis for depth of damage to dermis, blood perfusion to the burn using repeat LDI scanning, and time to re-epithelialise. Day 3 post-burn was considered to be representative of the deepest extent of dermal injury and the preferred time point for analysis relating histological depth of damage to time to re-epithelialisation. Updated time and temperature threshold data provided by this study was compared to previous estimates established by Moritz and Henriques in the 1940s (1).

In this study, a spill/splash event with water less than 85°C was shown to be unlikely to result in a severe scald injury which takes longer than 3 weeks to fully re-epithelialise. These results are in disagreement with injury prediction estimates from the 1940s which reported that a severe 2–3º scald would result from exposure to 60°C for 5 seconds and 70°C for 1 second (1). Here, by day 3, 60°C water for 5 seconds showed no histological evidence of injury to the dermis and 70°C for 5 seconds had damage to less than 25% of the dermis, indicating a superficial dermal injury. In addition to this, 5 second scalds with water up to 80°C were observed to have fully re-epithelialised within 3 weeks as did 70°C for 15 second scalds. It is possible that the differences in pig size between the 1940s study and this study (8–10kg compared with 27kg here), pig skin thickness (histologically assessed dermal thickness of 1–2mm compared with 2.04 ± 0.30mm here) and the anatomical location of burns may have contributed to these disparate findings. Results from a small pilot study conducted by the authors using 10kg pigs found their skin thickness was 1.40 ± 0.14mm (assessed histologically) (189). Unpublished tissue injury severity data from the pilot study supports findings that changes in the thickness of the skin with age and/or anatomical location may affect thermal injury susceptibility. For the same burn conditions, the younger pigs (10kg) showed deeper dermal damage than the older pigs (27kg). However, with 10kg pigs, 65°C water for 5 seconds was observed to result in evidence of only 29% depth of dermis damaged at day 3, which still does not equate to the 3º (full thickness) injury reported by the Moritz and Henriques study (1). Using current standards for histological classification
of burn severity and clinical evidence of time to re-epithelialise, results from this study clearly demonstrate that the time and temperature injury prediction data for a ‘severe deep’ burn presented in the 1940s by Moritz and Henriques (1) overestimates the expected burn severity.

Additional work recently published by the authors demonstrated that biopsy and processing procedures affect skin thickness measurements, with in vivo measurements being thinner than histologically assessed values (230). This explains the discrepancy reported here between ex vivo total skin thickness of 2.09 ± 0.20mm (assessed histologically) and the average in vivo skin thickness of 1.42 ± 0.23mm (determined using ultrasound) for the pigs (27kg) used in this study. Importantly, based on ultrasound measurements, the skin thickness of the pigs used in this study is similar to that reported for human children (94). An additional advantage to using pigs of this size (27kg) is their lateral thorax provides a large, relatively flat area with uniformity of skin thickness to allow for multiple burns to be created.

The threshold temperature of 85ºC for a clinically relevant deep dermal spill/splash injury determined in this study is comparable to calculated values recently presented by Abraham et al. using both an idealised computational model (78) and a numerical model (79) to estimate the time and temperature threshold for DDPT burns. Additional studies by Abraham et al. describe 82ºC as the approximate threshold temperature for which a DDPT burn may be predicted to result from a hot beverage spill event (79). However, it is very important to highlight that results from this study are not intended to replace threshold estimates for minor cutaneous thermal injury (superficial burn) (1). This study provides evidence-based injury prediction data to guide regulators setting standards for severe deep dermal injuries (which require extensive treatment), for example, the serving temperature of hot beverages and delivery temperature of ‘boiling’ water taps.

In countries such as Australia where the temperature of hot water delivered to sanitary fixtures (bathroom taps) is regulated, compliance to regulations is often poor (231, 232). As a consequence, although the incidence of hot tap water scalds has reduced, many children remain at risk of sustaining a severe scald injury from immersion in hot tap water (233). Additionally, a review of inflicted scalds in children by Maguire et al. (19) determined immersion injuries caused by hot tap water were more likely to represent child abuse compared to other types of burn injuries. In this present study, a statistically significant
relationship between increasing duration of exposure and increasing depth of dermal damage was observed for 55°C and 60°C water scalds. A child immersed in 50°C water for 10 minutes would be likely to sustain a moderate severity (mid-dermal) burn predicted to fully re-epithelialise within 3 weeks; exposure to 55°C water for 2 minutes would result in a similar severity burn; and immersion of 5 minutes in 55°C water would result in a severe burn likely to require surgical intervention. These findings provide strong evidence to support existing hot tap water safety guidelines and highlight the importance of compliance as just a 5°C increase in temperature contributes significantly to increasing the risk of sustaining a severe burn. Importantly, these results provide valid, evidence-based injury prediction data which, in conjunction with experienced clinical observations, can strengthen ‘expert opinions’ regarding burn injury severity for medicolegal proceedings where an inflicted hot tap water scald is suspected. Although it is imperative to note that absolute thresholds reported here are limited to porcine skin, equivalent contemporary studies for human skin are not available. Notably, this data updates previously accepted and legally cited estimates which were also established by porcine studies (1). While it was not feasible here to test for every burn condition a child may encounter, it is anticipated that in the future the data provided by this study will be used to develop and inform more comprehensive mathematical injury prediction models for immersion scalds.

This study found that a thermal injury to 75% of the depth of the dermis or deeper is required to result in a DDPT burn which is unlikely to fully re-epithelialise by 3 weeks. In a research setting, thickness classification of depth of burn is recommended (31, 55). However, evidence for the threshold value (percentage of the total dermal thickness damaged) which will result in a severe deep dermal injury not fully re-epithelialised by 21 days, is scarce. Using a porcine contact burn model, Renkielska et al. proposed that when less than 60% of the dermal thickness was damaged, a burn was likely to heal within 3 weeks (124). However, their study was comparatively small (23 wounds analysed) and only high temperature contact burns (80–90°C) were investigated. Ponticorvo et al. also used a high temperature (100°C) contact burn model and categorised deep partial thickness burns as those involving 35–65% of the thickness of the dermis (based on identification of collagen coagulation with Mason Trichrome stain) and full thickness burns as those deeper than 65% (146). However, Ponticorvo et al. did not follow any burn conditions to re-epithelialisation. This study utilising vascular damage as a marker of tissue injury proposes a higher relative injury to the dermis of 75% or deeper to represent a DDPT scald burn which is predicted to not fully re-
epithelialise by day 21. When tissue damage extends to less than 75% of the dermis it is plausible that although significant damage to the vessels of the dermis may result, there are still enough functioning vessels deeper in the dermis to supply blood to the adnexal appendages and support re-epithelialisation. While this threshold was consistent for the majority of burns, histological depth of injury for the 85°C for 5 second burns showed damage to the dermis of less than 75% at day 3 but did not fully re-epithelialise by 3 weeks. The reason for this remains unclear, however one explanation is that for 5 second duration scalds, water at 85°C is close to the temperature which distinguishes between a burn which will or won’t fully re-epithelialise by 3 weeks and as such the relative error due to any biological or methodological variation may have a greater influence on burn depth. Indeed, overall variation between replicates for depth of damage was greater for short duration (5 second) spill/splash scalds than immersion scalds. For burn models where a pre-defined and reproducible depth of injury is desired, these findings support recommendations by others (44, 180) to preferentially use burn conditions involving longer exposure durations (and correspondingly lower temperatures) to minimise any relative errors in exposure time which may influence burn depth.

Due to concerns regarding disruption to wound healing, the same individual burn was not assessed histologically and subsequently followed for time to re-epithelialise, which is a major limitation of this study. Additionally, there is contention over agreement between gross macroscopic evaluation and histological assessments for re-epithelialisation (155). While good agreement between techniques was observed here, given the majority of the wounds had healed, this is not unexpected. Therefore while provision of a quantitative threshold value (75%) for depth of dermal damage to objectively assess whether burns will fully re-epithelialise or not improves understanding of how histological damage relates to re-epithelialisation outcome, further work in this area is recommended. In particular, it is unknown how well this threshold may perform for human skin, other mechanisms of thermal injury such as contact burns, and where different dermal elements are identified as tissue injury markers.

Recently our group conducted a systematic review of porcine burn models and noted a substantial discrepancy between scald and contact burns for the burn conditions reported to create DDPT injuries (234). Results from these experimental studies support those findings, showing burn conditions for DDPT scald injuries involve comparatively lower temperatures and shorter exposure times than those reported for contact burns. For
example, 90°C water with an exposure time of 5 seconds was observed to result in damage to 48% of the depth of the dermis (histologically determined at 1 hour post-burn). However, double this exposure time (10 seconds) is reported to cause an equivalent depth of dermal injury (50%, endothelial cell injury at 30 minutes post-burn) from contact with a heated Aluminium bar (55). Similarly, here at 1 hour post-burn, 60°C water for 30 seconds showed damage to 53% depth of the dermis, compared to only 15% depth of endothelial cell injury (at 30 minutes post-burn) reported for a contact burn with equivalent burn conditions (55).

Possible explanations for these differences include variance in thermal conductivity, the high specific heat capacity of water compared to most materials used to create contact burns, and an enhanced rate of heat exchange for liquids in motion compared to solid materials. Additionally, unlike a pre-heated metal bar, water in this study was refreshed and maintained at a consistent temperature throughout the exposure. This is important as known patterns of injury from bathroom hot water taps include running water (235).

Clinically, differences in the nature of scald and contact burns are perceived. However, it remains difficult to speculate on whether differences described for contact and scald injuries are due to altered pathophysiology or to disparities for intensity of the heat and duration of exposure. For high temperature contact burns Brans et al. (37) speculated that immediate coagulation created a layer of necrotic tissue (observed on histological examination) which could function as a barrier to further heat conduction, reducing damage to the deeper vascular structures of the dermis. Such clear delineation in collagen damage was not observed for any of the high temperature scald injuries tested here. Indeed, for the moderate severity injuries shown here there was substantial intermingling of both blocked and patent vessels, indicating for these burns superficial vessels could remain undamaged even though injury to the deeper vascular structures was apparent. For immersion scald injuries with low intensity heat and short exposure times (50°C for 1–5 minutes and 55°C for 1 minute), an initial hyperaemic response was observed histologically to result in more transient congestion of vessels. As exposure times increased, the congestion of vessels extended deeper within the dermis and was more likely to be associated with irreversible damage. A better understanding of the exact in vivo tissue temperature at which tissue damage occurs is required to further appreciate differences between scalds and contact burns.

There are many external factors known to affect the reliability of LDI scanning results. For example, it is well recognised that burn debris, blisters, and necrotic epidermis can give a false impression of low perfusion by reducing the reflected laser light (132, 133). However,
there is little information on how repeat sampling with full thickness biopsies may disturb the wound and affect LDI perfusion results of scans at later time points. In this study, despite exclusion of prior biopsy sites from perfusion calculations, perfusion levels in previously biopsied burns showed increased variation between replicates and were significantly higher than those from equivalent burns with no biopsies (performed at the same time post-burn). This effect was greater as more biopsies were taken (i.e. by day 7) and similar for both moderate and more severe burns. Therefore, caution is recommended when interpreting LDI perfusion results from burns which have previously been biopsied. Additionally, these findings combined with clinical observations of increased erythema and excoriation in wounds which have had biopsies, suggest where repeat full thickness biopsies are taken from a relatively small burn area, disturbance to the wound may extend beyond the biopsy margins. The possible impact this may have on wound healing overall was not investigated and remains unclear. For future studies in this area, a suggested improvement in study design includes creation of two equivalent burns on the same pig, where one burn is used for LDI and gross visual re-epithelialisations evaluations and the other for repeat biopsies for histological investigations, including microscopically determined re-epithelialisation.

Contrary to human studies (121, 132, 236), but in agreement with other porcine studies (127), a single LDI scan at day 3 post-burn was not observed to be predictive of the healing outcome of moderate to severe burns. Perfusion values at day 3 for moderate severity burns which fully re-epithelised by day 21, were not significantly higher than for burns which did not fully re-epithelialise. In our study, LDI results at day 7 showed improved ability to distinguish DDPT burns from more moderate severity burns, suggesting a later peak in accuracy of LDI scans for burns of this severity. Furthermore, repeat measurements improved the strength of LDI predictions, with DDPT burns (not fully re-epithelialised by day 21) showing little increase in perfusion from day 3 to 7. Similar observations are reported by Stetinsky et al. (137) in a prospective human study performing repeated LDI scans over the first 9 days post-burn and comparing these to healing times. Similar to a biopsy, a single LDI measurement provides only a ‘snap-shot’ of dermal blood flow at a given time point and where feasible, sequential measurements at later time points are recommended to better predict healing outcomes.

This study was limited to investigating water temperatures of no greater than 90°C due to the thermal rating of the submersible pump and issues with distortion of the plastic tubing of the scald device at very high temperatures. However, a small number of scalds with very
high water temperatures (>90°C) were created using an alternative method; 40ml of water (in a 100ml Schott bottle) was heated in a microwave to the desired temperature and then poured into the device (instead of continuous supply of heated water from a water bath). The alternative method used to create these spill/splash (5 second duration) scalds resulted in comparatively lower and less consistent water temperatures within the device and were therefore excluded from analysis. In future, for testing of water higher than 90°C, it is recommended that shorter exposure times of 1–2 seconds be investigated using an alternate method.

6.6.1 Limitations

The advantages of using a porcine model for thermal injury investigations are well known, however, no animal model is a perfect representation of the human situation. While in this study the translatability of results to a child’s skin is enhanced by similarity in dermal thickness, there are many other biological and anatomical variables which may affect an individual’s susceptibility to thermal injury. Generalisation of results from porcine skin to human skin remains a major limitation of this study, and recommendations informed from these experimental animal studies would be strengthened if supported by evidence from clinical observations in children. Additionally, the healing capacity of a juvenile pig housed under experimental conditions may differ from that of a human child. Furthermore, in a real-life situation, there are numerous different scenarios by which an actual scald injury can occur, which may be quite different to the standardised and controlled burns created for this study. It should also be noted that no first aid or treatments were applied to any of the burns. Therefore the conditions for deep dermal injury results presented here are likely to represent the ‘worst case scenario’ for a human child exposed to a similar scald injury event. For development of burn injury prevention guidelines where the aim is to reduce the risk of severe burn injuries and for informing medicolegal judgements, this approach is valid and the data presented here is invaluable evidence for these guidelines.

Additional limitations of this study include: although a large number of burns were created, given the broad range of experimental burn conditions the number of replicates for each condition was relatively small; for re-epithelialisation investigations the study duration was limited to 3 weeks and observations regarding time to fully re-epithelialise for all burns and final scar outcome were not possible; and while dichotomous categorisation of burns into those which had or hadn’t fully re-epithelialised by 3 weeks is clinically relevant, it is less
sensitive than a continuous analysis of re-epithelialisation data e.g. days to complete healing. Finally, to minimise animal use, priority was given to investigating healing of deep dermal scald injuries, however, for completeness, inclusion of more burn conditions causing severe full thickness injuries may have been considered.

### 6.7 Conclusions

A revision of the scald injury prediction data for the burn conditions expected to result in a clinically relevant deep dermal burn is long overdue. Using a porcine scald model, evidence-based data produced from this study contributes significantly to improving understanding of the relationship between water temperatures, duration of exposure and tissue injury severity. The time and temperature thresholds for deep dermal injury established here in a porcine model represent the ‘worst case scenario’ for a human child exposed to a similar scald injury event and can be used to guide burn injury prevention strategies and legislation. Additionally, as an adjunct to clinical observations, this data is a valuable scientific resource for clinicians considering cases where an inflicted mechanism of scald injury is suspected.

### Acknowledgments

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**Conflicts of interest:** All the authors have no conflict of interest.
6.8 Chapter Conclusion

The research study presented in this chapter clearly demonstrates for the first time, that existing predictions for deep scald injuries, reported in the 1940s, overestimate the expected burn severity. Importantly, updated time and temperature relationships for severe scalds are established. As this study was limited to porcine skin, for human skin these thresholds should be considered as a guide only and represent conservative, ‘worst case scenario’, estimates for a human child exposed to a similar scald injury event.

As a result of this study, these updated scald injury prediction data are now used by clinicians at Queensland’s major paediatric burn treatment centre to inform police investigations where an inflicted mechanism of scald injury is suspected. It is anticipated the published data from this work may also be cited in legal proceedings, replacing the outdated injury prediction estimates from the 1940s (1).

The animal studies reported in this chapter provide strong experimental evidence for the water temperatures and durations of exposure for severe scald injuries. It is now necessary to consider validation of these findings for human scald injury circumstances. Specifically, it is of interest to evaluate the translation of these findings to scald injuries in children. The next chapter addresses this issue and describes a study undertaken to assess the burn conditions reported for severe scalds in Queensland’s children.
Chapter 7  Burn conditions reported for severe scalds in Queensland children

This chapter is not based on any published works
7.1 Chapter foreword

The main findings and recommendations from the experimental animal studies were presented in the previous chapter ((chapter 6, (237)). The purpose of this chapter is to review the burn conditions reported for severe scald injuries in children for comparison.

The study presented in this chapter explored the aetiology of severe scald burns in Queensland children. A retrospective chart and database review was conducted to collect information regarding the burn conditions and circumstances for children treated at Queensland’s major paediatric burn treatment centre.

While no part of this chapter has been published it will presented to burns clinicians at the Australian and New Zealand Burn Association (ANZBA) Conference, Adelaide, October 2017.
Burn conditions for severe scald injuries requiring split-thickness skin grafting (SSG) in Queensland children

7.2 Introduction

Each year over 1000 children with burn injuries present to Queensland’s major paediatric burn treatment centre, the Pegg Leditschke Children’s Burns Centre (PLCBC). Scald as a mechanism of burn injury in children is very common, accounting for around half of the burn injuries treated at PLCBC (233). While all scald injuries are painful and distressing for a child, severe burns which require surgical intervention and extensive treatment also carry a high risk of scarring, which has lifelong physical and psychological implications (3, 238).

Although scald injuries are a significant paediatric public health issue, the burn conditions (water temperature and duration of exposure) to result in a severe scald, which is likely to require surgical intervention, are unknown. International approaches to hot water safety are based on scald injury prediction data from the seminal work of Moritz and Henriques published in the 1940s (1). The limitations of Moritz and Henriques study and the uncertainty regarding the validity and of their findings for severe scald injuries has been discussed in detail throughout this thesis (chapters 1, 3 and 6) and by others (48, 239). Experimental evidence from the porcine scald injury study, presented in the preceding chapter (chapter 6, (237)), shows the time and temperature threshold data from Moritz and Henriques study (1) overestimates injury severity for severe scalds. However, it remains unclear if these findings, from the experimental animal studies, translate to outcomes in human skin. Recommendations informed from the experimental animal studies presented in this thesis, would be strengthened if supported by evidence from clinical observations in children.

Clinically, burn injuries assessed by an experienced clinician as DDPT or FT are considered unlikely to fully re-epithelialise in 2–3 weeks and more likely to result in hypertrophic scar formation (3, 5, 6). For improved healing outcomes, surgical intervention with split-thickness skin grafting (SSG) is indicated as a treatment for severe deep burns which are predicted to take longer than 3 weeks to heal (5). Anecdotally, burns clinicians at PLCBC have perceived that injury prediction data for severe deep scald injuries provided by the 1940s studies, does not equate with their clinical observations. For example, based on Moritz and Henriques data, it is inferred that exposure to 60ºC water for 5 seconds will result in a child sustaining a severe FT burn (1, 240). However, clinicians at PLCBC observe these burn conditions are
generally more consistent with a less severe, SPT injury. There is scant clinical evidence available from the current burns literature to describe the exact circumstances of exposure for severe scald burns in children.

The aim of this retrospective chart review was to identify and characterise the circumstances of severe scald burns in children and to improve understanding regarding the burn conditions (water temperature and duration of exposure) required for a child to sustain severe scald injuries which require grafting.

7.3 Materials and Methods

This study was a retrospective database and chart review. The study population was from Queensland’s major paediatric burn treatment centre. Prior to November 2014 this was the Stuart Pegg Paediatric Burns Centre (SPPBC) located at the Royal Children’s Hospital (RCH) in Herston, Brisbane. The centre was relocated to the Lady Cilento Children’s Hospital (LCCH), South Brisbane in November 2014 and is now the Pegg Leditschke Children’s Burns Centre (PLCBC).

Ethics approval for this study was obtained from the Queensland Children’s Health Services Human Research Ethics Committee (HREC/16/QRCH/393). Children (under 18 years old) who presented to the burns centres (SPPBC and PLCBC) for treatment of their scald burn injury from 1st January 2013 to 11th November 2016 were identified from the Queensland Paediatric Burns Registry (QPBR). The QPBR is a database developed by the Centre for Children’s Burns and Trauma Research (CCBTR) to collect patient data at the time of presentation from both inpatients and outpatients using a detailed pro forma with parental/guardian consent. All children identified on the pro forma as sustaining a ‘scald burn’ from water, hot beverages or steam were included, scalding from hot oil and foods such as noodles and soup were excluded.

Data collected included: child demographics (age, gender), injury event details and descriptions, burn depth and total body surface area (TBSA) of burn, and operative management with split-thickness skin grafting (SSG).

Children identified as having a scald injury treated with SSG were further evaluated. Additional information regarding the burn conditions (estimated water temperature and duration of exposure) was collected including type of fluid involved, time from preparation,
and whether milk had been added to hot beverages. Where information provided in the QPBR database was incomplete/inadequate a review of the medical charts for information regarding the specific burn conditions was conducted. For a `spill/splash’ scald event, the exact duration of exposure was not identifiable from carer descriptions and was considered likely to be ≤ 10 seconds (to include time to remove wet clothing). Estimates for water temperature were based on the information that was collectively most consistently reported and included: for hot beverage scalds (HBS), whether or not the beverage was freshly made (≤ 1 minute) and whether milk had been added or not; for scald events involving kettles, saucepans or other containers, whether the water was boiling or had recently boiled (≤ 1 minute). Immersion scald events usually involve a more sustained exposure to hot water and were identified by exposure times of >10 seconds. If available, the temperature of water delivered to domestic hot taps was recorded. For hot tap water scalds it was noted whether the home had a tempering valve installed or not.

Data analysis was performed using GraphPad Prism V6 (GraphPad Software, Inc. California, USA).

7.4 Results

7.4.1 Circumstances of all scald injury events

A total of 3239 children were treated for burn injuries at the SPPBC and PLCBC between 1st January 2013 and 11th November 2016. Of these, 45% (n = 1465) recorded ‘scalding’ as the mechanism of injury, which included scalding from hot water, hot beverages, steam, oil, or food (e.g. noodles, soup). After excluding scalding from oil and food (n = 465), there was a total of 1000 children treated for scald injuries included for analysis. HBS were identified as the leading scald injury event accounting for over half of the cases (52%) and spill/splash scalds from a saucepan/kettle comprised a further 23% of cases (Table 7-1). Bathroom scalds from hot tap water (water from tap/bath/basin/shower) accounted for only 6% of cases.
Table 7-1 Circumstances of injury event for all hot water, hot beverage and steam related scald injuries (n = 1000)

<table>
<thead>
<tr>
<th>Scald injury event</th>
<th>Cases (n)</th>
<th>% of total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hot beverage</td>
<td>519</td>
<td>52%</td>
</tr>
<tr>
<td>Water from saucepan/kettle</td>
<td>234</td>
<td>23%</td>
</tr>
<tr>
<td>Water from bucket/container</td>
<td>81</td>
<td>8%</td>
</tr>
<tr>
<td>Water from tap/bath/shower/basin</td>
<td>63</td>
<td>6%</td>
</tr>
<tr>
<td>Steam vaporiser</td>
<td>42</td>
<td>4%</td>
</tr>
<tr>
<td>Steam from another source</td>
<td>19</td>
<td>2%</td>
</tr>
<tr>
<td>Water from hot water bottle</td>
<td>19</td>
<td>2%</td>
</tr>
<tr>
<td>Other</td>
<td>16</td>
<td>2%</td>
</tr>
<tr>
<td>Unknown</td>
<td>7</td>
<td>1%</td>
</tr>
</tbody>
</table>

7.4.2 Circumstances of severe scald injuries requiring SSG

Of the 1000 scald injury cases identified, 25% (n = 246) were assessed on presentation by clinicians to be DDPT or FT burns (Figure 7-1). However, only 47 children (4.7%) underwent SSG surgery. Of these, 4 cases were excluded from further analysis as 2 cases had concurrent friction burn injuries, 1 case had an incomplete pro forma which was also de-identified (therefore a chart review was not possible), and 1 case was a steam burn (not hot water). Thus, a total of 43 cases of severe scald injuries (requiring SSG) were included for further analysis. Immersion scalds (water from tap/bath/shower/basin) accounted for only 9% (n = 4) of cases. The majority of SSG cases arose from a spill/splash event (Table 7-2). Males were over represented, accounting for 60% of cases and the average age was 39 months (range 1–174 months).

The median TBSA of all scald injuries treated with SSG was 5% (95% CI: 3–7%). Both HBS and water from saucepan/kettle scalds had a similar median TBSA of 5% and 6.25% respectively, while water from a bucket/container scalds had the highest individual TBSA of 40% (median 5%). The majority of incidents (79%, n = 34) occurred in the usual residence (home).
Figure 7-1 Flow chart of cases included for severe scald injuries requiring split-thickness skin grafting (SSG).

Table 7-2 Circumstances for all severe scald injuries requiring SSG (n = 43)

<table>
<thead>
<tr>
<th>Burn conditions</th>
<th>Hot beverage scalds</th>
<th>Water from saucepan/kettle</th>
<th>Water from bucket/container</th>
<th>Water from hot water bottle</th>
<th>Water from tap/bath/shower/basin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Spill/splash (≤ 10 sec)</td>
<td>17</td>
<td>14</td>
<td>5</td>
<td>3</td>
<td>0</td>
</tr>
<tr>
<td>Immersion (&gt; 10 sec)</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>4</td>
</tr>
<tr>
<td>Freshly boiled (≤ 1 minute)</td>
<td>12</td>
<td>12</td>
<td>3</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Not freshly boiled (&gt; 1 minute)</td>
<td>2</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>n/a</td>
</tr>
<tr>
<td>Unknown</td>
<td>3</td>
<td>1</td>
<td>1</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>Temperature of hot tap water known</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>n/a</td>
<td>1</td>
</tr>
</tbody>
</table>
7.4.2.1 Estimated water temperature and duration of exposure for severe scalds

Of the 4 cases involving immersion in water from a domestic hot tap, the burn conditions could only be reliably estimated for one case. In this case, the child was reported to have been immersed in 62°C water (temperature of water delivered to hot tap measured after the event by a plumber) for approximately 1–2 minutes (caregivers estimate). None of the homes were reported to have tempering valves, therefore water temperatures in excess of 50°C were probable for all cases.

Of the cases categorised as spill/splash events, the duration of exposure most commonly reported was just a few seconds, however, when allowing for removal of wet clothing, exposure times increased to 10 seconds. Overall, for spill/splash scalds, there was insufficient information collected to enable estimation of the burn conditions for 21% (n = 8) of cases (Table 7-2). For HBS, burn conditions could be estimated for 14 cases (Figure 7-2); in the majority of cases (86%, n = 12), the beverage was described as being recently made (≤ 1 minute). Additionally, for the 14 cases of HBS where burn conditions could be estimated, addition of milk to the beverage was not described for any cases, the majority (71%, n = 10) had no milk added, and this information was unknown for the remaining 4 cases. There were only 2 cases of HBS where the beverage was not freshly made, for both these cases black tea made approximately 2–5 minutes prior to the spill/splash event was described.

For water from a saucepan/kettle scalds, where burn conditions could be estimated, all but one case involved boiling or freshly boiled (≤ 1 minute) water. That one case involved water from a kettle that had been boiled approximately 5 minutes earlier. For water from a bucket/container scalds: in one case no estimates could be made, in 3 cases the water was described as freshly boiled (≤ 1 minute), and in one case the water had been boiled and then poured into a Tupperware container for steam inhalation approximately 4 minutes prior to the scalding event. Finally, no estimates for water temperature could be made for all 3 cases where scald injury from a leaking hot water bottle was reported. Overall, for spill/splash events, the burn conditions could be estimated for 31 cases. Of these cases for which the burn conditions could be estimated, 87% (n = 27) were from water that was boiling or had recently been boiled (≤ 1 minute).
7.5 Discussion

As anticipated, scalding as a mechanism of burn injury was common, accounting for 45% of the paediatric burn injuries seen at SPPBC and PLCBC during the study period. Over the four year study time period (2013–2016), 47 children in Queensland (equivalent to approximately 10 per year) had SSG surgery for their severe scald injuries. Although the number of children requiring SSG represented only a small proportion of the cases presenting with scald injuries, patients with severe burns which require grafting are at greater risk of developing hypertrophic scarring (HTS) (238). Scarring can be a particularly problematic and devastating outcome for a growing child. This study aimed to enhance understanding of the burn conditions for these severe injuries, as this information is crucial to better inform burn injury prevention measures and improve health outcomes for children.

From the retrospective analysis of collected data, it was not possible to accurately identify the precise water temperatures or durations of exposure involved. However, from the information available for spill/splash scalds, it was possible to group injury events into those involving boiling or recently boiled (≤ 1 minute) water or those involving water which had not
recently boiled (> 1 minute). Additionally, for HBS, estimates were further refined by considering whether milk had been added to the beverage or not. The addition of milk to a hot beverage such as tea or coffee has been shown to shorten the cooling time (241, 242). A study by Jamnadas-Khoda et al. (242) demonstrated that black beverages had higher starting temperatures (87–88°C) compared to milky beverages (75–77°C). Importantly, they also determined that 1 minute after a hot beverage had been prepared, only black beverages (no milk added) remained above 80–85°C. It is understandably difficult to obtain reliable and precise information from anxious parents and caregivers regarding the water temperatures involved in the scald event at the time of presentation to the burns centre. However, by having an understanding of the cooling rates involved and grouping cases accordingly, it was possible to refine estimates for the water temperatures involved.

Similar to previous investigations (17), in this study, HBS continue to be the leading cause of scald injuries in Queensland children. Given the importance of HBS as a mechanism of scald injury, it is important to consider whether lowering the serving temperature of hot beverages would reduce the risk of a child sustaining a severe deep scald injury. Guided by Moritz and Henriques estimates from the 1940s (1), to reduce the risk of a child sustaining a severe HBS, it would be necessary to decrease the serving temperature to less than 60–65°C (242). Whilst temperatures in this range may limit the potential for significant scalds, they may be considered unsatisfactorily low for consumers of hot beverages. Here, there was little evidence to suggest that temperatures below 80°C were associated with severe scald injuries which require grafting. Instead, almost all of the HBS involved beverages which had been recently made (≤ 1 minute) with no added milk, which suggests the temperature of the scalding liquid was 80°C or higher (242). Therefore, to reduce the risk of a severe scald injury, a serving temperature of ≤ 80°C is recommended as a more satisfactory and achievable benchmark.

Interestingly, while overall HBS accounted for over half (52%) of the scald injuries treated, they only represented 40% of the cases which required grafting. While this is still a large number of cases, it is proportionally less than may have been anticipated. Conversely, hot water from saucepans/kettles was implicated in fewer (23%) scald injuries overall, but accounted for 33% of cases which required grafting. One possible explanation for these findings is that a greater volume of hot water is more likely to be involved in spill/splash scalds from kettles/saucepans, resulting in a larger burn area. However, this study shows the median ± SD TBSA for HBS and kettle/saucepan scalds was similar, 5 ± 6.5% and 6.3
± 5.8%, respectively. It is more plausible that the temperature of the scalding liquid from a kettle/saucepan is hotter than that for most hot beverages. A study by Mercer (241) investigated the cooling characteristics of hot water containers, finding that the larger volume of fluid accommodated by kettles/saucepans reduces the cooling rate of the liquid. The author reported that for a plastic kettle containing 1.5 litres of water, 1 minute after boiling the water was still a high 97°C and by 10 minutes this had only reduced to 84°C. Here, for SSG cases where water from a saucepan/kettle was involved and burn conditions could be estimated (n = 13), the water had been recently boiled (≤ 1 minute) in almost all cases (n = 12). For the remaining case, the kettle was reported to have been boiled approximately 4 minutes prior to the scald event. These circumstances for kettle/saucepan scalds further support findings that for children, severe scald injuries requiring grafting do not commonly involve temperatures below 80–85°C.

The majority of incidents of severe scald injury occurred in the child’s usual residence (79%). Therefore, as previously suggested (17), priority should be given to public education campaigns designed to increase awareness regarding the danger posed by hot liquids in the home. While emphasis is rightly placed on educating caregivers regarding the risk of HBS, here, hot water from a recently boiled kettle/saucepan was also implicated in a substantial proportion of SSG cases. Overall, the rate of grafting for kettle/saucepan scalds was 6%, which is much lower than other studies reporting kettle scalds in children: a Norwegian study reported out of 29 cases, 52% needed skin grafting (243); and a more recent prospective South African study found that of 119 kettle burns, grafting was undertaken in 52% (244). However, data sets from these other studies may have been heavily skewed by including only the number of children who were hospitalised for their injuries (in-patients). Here, over the study time period, only 50 children were treated as in-patients for kettle/saucepan scalds and SSG was performed in 28% (n = 14) of these. Additionally, differences in grafting rates may also be attributable to variances in treatment approach (between burns centres) for severe burns e.g. early or delayed excision and skin grafting. Nonetheless, given the large number of kettle/saucepan scald injuries which are severe and require grafting, it is important to advocate for improved preventative measures to reduce the incidence of this mechanism of injury.

Despite prevention strategies and legislation in Australia designed to minimise the risk of domestic hot tap water scalds, compliance with regulations is inadequate (232, 233). Overall, hot water from tap/bath/shower/basin accounted for 6% (n = 63) of all scald injuries,
which is consistent with other recent studies of paediatric burn admissions in the UK and US, reporting rates of 7–11% (16, 235). In the US study by Shields et al. (235), which reviewed the National Electronic Injury Surveillance System in the US for scald burn incidence in children under 3 years of age, children aged 1–5 months were most likely to have sustained their scald injuries associated with bathing (49.6%, n = 64). The danger posed by hot tap water is further highlighted by findings from a single burn centre review by Baggott et al. (245), where 6% (n = 56) of all paediatric scald burns admissions (0–5 year old children) were from hot tap water while sink-bathing, and 11% of these patients required skin grafting. It is therefore concerning that in a recent Queensland study, where 17 families completed a tempering valve survey at the time of their child’s bathroom scald injury, only 2 homes reported having tempering valves (233). In the present study, all 4 cases of severe hot tap water scalds requiring grafting occurred in residences with no tempering valves installed. Tempering valves are designed to limit the temperature of the water delivered to sanitary fixtures (hot taps) to 50ºC (214). Predictions from thermal studies of the 1940s suggest that for 50ºC water exposure times of ≥ 5 minutes would be required to cause a severe (DDPT to FT) burn (1, 240). Here, in one case, water from the hot tap was measured to be 62ºC. For this case, if tempering valves had been in use (water ≤ 50ºC), the 1 to 2 minute duration of exposure described by the caregiver would have been unlikely to result in the child sustaining a severe burn. Clearly, compliance with safety measures such as tempering valves remains an issue and increased awareness is needed regarding the danger posed by water in excess of 50ºC from hot taps.

Unfortunately, the small number of SSG cases reporting hot tap water immersion as the mechanism of injury limited estimations for the burn conditions to result in a severe scald injury. Compared to the spill/splash scalds, it was more difficult to define approximate exposure durations and possible water temperatures involved from the available information for immersion scalds. Given the small number of cases involved, it is feasible to recommend that in future the temperature of the water delivered by the hot tap be measured for every case of severe scald injury from hot tap water treated at PLCBC. This would potentially make the home safer for the family involved and also enable a more comprehensive understanding of the burn conditions involved in immersion scald events for future analysis.

This study was retrospective in design and therefore the scope of the study was limited by the availability of information for the burn conditions involved for all cases. Despite additional chart reviews, the burn conditions were unable to be estimated for 25% of the cases of
severe scalds where grafting was required. Inconsistencies in quality of data entered into the database was also a concern. Additionally, estimates for water temperature and duration of exposure were subjective and relied upon histories given by patients and caregivers at the time of presentation, when accurate recall of the scald injury event can be difficult. Improvements to the pro forma have been recommended including collection of more specific information regarding timing and details of the scald event e.g. length of time after the kettle/saucepan was boiled or the hot beverage made, and ensuring information regarding tempering valves is collected for all cases of hot tap water scalds. SSG was chosen as the outcome measure to identify the most severe scald injuries, however, the requirement for SSG may be influenced by factors other than the initial severity of the burn, such as co-morbidities and anatomical location. Additionally, over recent years, following the introduction of new silver-impregnated dressings at PLCBC, a decrease in the number of burn injury cases treated with grafting has been noted (17).

7.6 Conclusion

This research improves understanding regarding the severity of, and the mechanisms by which, scald injuries occur. Scald injuries from a spill/splash with liquid from a recently made hot beverage (≤ 1 minute since made) or recently boiled kettle/saucepan (≤ 1 minute) were the most common mechanisms by which severe burns requiring SSG occurred. Contrary to data from the 1940s, but in support of findings from the experimental animal studies in this thesis ((chapter 6, (237)), a short duration of exposure to water less than 80–85°C is unlikely to result in a severe scald injury which will require surgical intervention. This study also highlights the importance of compliance to domestic hot tap water safety measures, finding that no tempering valves were installed in the homes in all cases of severe scalds from hot tap water. Data from this study can be used to inform and target scald injury prevention measures designed to minimise the risk to a child of sustaining a severe scald injury.


7.7 Chapter Conclusion

The research study presented in this chapter has shown that scald injuries in children occur through a broad range of mechanisms and circumstances. This study demonstrates that although no two injuries are the same, it is possible to obtain meaningful data regarding the water temperature and duration of exposure for severe scald injuries in children. However, the strength of this data would be greatly enhanced if the consistency of the scald injury data collected was improved. As a result of this study, several of the recommendations to improve data collection at PLCBC have been applied. In particular, for scald injury events involving domestic hot tap water, greater emphasis has been placed on collecting information regarding tempering valves and estimating duration of exposure.

In the absence of equivalent experimental data for thermal injury in human skin, this observational paediatric data provides valuable clinical evidence to validate findings from the experimental animal studies presented in this thesis (chapter 6). A summary of the main findings and of the principal issues and recommendations which have arisen from this research project are provided in the next chapter, which is the final chapter of this thesis.
8.1 Overview

Scald injuries are common in early childhood and are a serious global paediatric public health problem. Clinically, deep burns predicted to take longer than 3 weeks to heal (re-epithelialise) are of particular importance as these injuries are likely to require admission to hospital for extensive treatment, may require surgical grafting and are more likely to scar. To develop effective scald injury prevention guidelines targeted at reducing the severity of injury, it is essential to have valid evidence-based injury prediction data. However, the burn conditions (water temperature and duration of exposure) predicted to result in a severe deep burn from a spill/splash or immersion scald event are unknown. For the first time, the work presented in this thesis provides definitive and compelling evidence to answer this question. Additionally, robust scientific evidence to inform medicolegal proceedings is presented and has already been used by clinicians to support evaluations in cases where inflicted hot tap water burns were suspected.

The main work in this thesis describes an experimental study using a porcine scald model to establish the time and temperature threshold for severe deep scald injuries (chapter 6). The multitude of data and tissue samples collected during both the creation of the burn model and whilst conducting animal experiments enabled development of other studies which form additional bodies of work produced for this thesis. Several novel concepts were examined, including:

- Determining the extent to which heat conduction through skin is affected by skin thickness, burn temperature, and burn duration (chapter 4).
- Evaluating the changes in skin thickness measurements with excision and biopsy processing procedures (chapter 5).
- Using Laser Doppler Imaging to examine the vascular changes in the skin within 7 days post-burn and relating this to wound healing (chapter 6).
- Assessing the reliability of histopathological staining approaches for determining pathological changes in cells and tissue damage after a burn injury (Appendix A).

The main findings from the studies in this thesis and how they relate to achieving the original aims of this research project are discussed below.
8.2 Main findings

8.2.1 Accurate injury prediction data for severe scalds is lacking and cannot be determined from analysis of existing studies or clinical observations alone

Invariably, any search of publically accessible scald injury prevention literature leads to data from the seminal work of Moritz and Henriques (1) in the 1940s. While the series of ‘thermal studies’ conducted by Moritz and Henriques (1, 33, 194) has undoubtedly provided crucial data to inform international scald prevention guidelines, for too long the validity of their results for predicting severe deep scald injuries has gone unchallenged. Anecdotally, many burns clinicians have perceived that estimates for the burn conditions to result in a deep dermal to full thickness scald injury (informed from the 1940s studies) do not equate with their clinical observations. This research project is the first study to comprehensively re-examine the relationship between burn temperature, duration of exposure and tissue injury severity for scalds.

This thesis has clearly demonstrated the limitations of previous studies for providing specific injury prediction data for clinically relevant severe scald injuries. The literature review (chapter 1) confirmed that approaches to evaluation of burn injury severity and burn wound healing have changed since the 1940s. Greater emphasis is now given to making treatment decisions based on distinguishing between SPT and DDPT injuries as a means of predicting the healing outcome (time to re-epithelialise) of a burn. The key weakness of Moritz and Henriques studies (1) is they do not provide quantitative histological data for depth of dermal injury, or data for time to re-epithelialise. Nevertheless, researchers developing mathematical models and computer simulations (74, 109, 246) to predict tissue damage from scald injuries are still reliant on this outdated data.

8.2.1.1 Systematic review of existing studies

Porcine burn models have been used extensively for a broad range of thermal injury investigations. Therefore, it was considered plausible that analysis of these existing studies could provide more accurate injury prediction data for severe scalds. Importantly, this would reduce the need for further animal experiments. This thesis included the first study to evaluate whether a pooled analysis of data from existing porcine burn models could be used to calculate the burn conditions required to create severe deep scalds. A systematic review including 42 studies found that DDPT burns were created using lower temperatures and shorter durations of exposure for scald burns (83°C for 14 seconds) compared to contact
burns (111°C for 23 seconds) (234). The review showed that the mechanism of injury, e.g. contact or scald, had an important effect on burn severity, suggesting caution where burn conditions established by contact burn models are used to guide scald burn prevention strategies.

The majority of porcine burn models included in the review created contact injuries. Only one out of the five included scald models reported a comprehensive range of scald burn conditions. However, this was the study by Moritz and Henriques (1) and the limitations of their methodological approach to evaluating depth of injury to the dermis have been discussed extensively throughout this thesis. Apart from the study by Moritz and Henriques (1), no other experimental porcine studies were identified which could provide strong, evidence-based injury prediction data, for the time and temperature threshold for a deep dermal scald injury. In particular, it remained difficult to estimate the severity of injury to the dermis from exposure to moderate temperature water (50–75°C), which is a clinically relevant temperature range seen with domestic hot tap water immersion scalds.

The systematic review of porcine burn models achieved its primary aim of providing a comprehensive qualitative and quantitative appraisal of the relevant literature. However, rather than refining estimates for injury prediction, the review demonstrated that major discrepancies exist between studies for the burn conditions reported to cause DDPT and FT burns. Even allowing for the use of different methods and materials to create burns, these differences are substantial. One of the key benefits of conducting the systematic review and assessing the quality of included studies was to reveal the methodological and outcome measurement strengths and weaknesses of existing experimental porcine burn models. This knowledge was essential for improving the design and optimising the novel porcine scald burn model developed for this thesis (Chapter 3).

8.2.1.2 Clinical observations alone cannot replace experimental animal studies

Another approach taken to evaluate the relationship between water temperature, duration of exposure and tissue injury severity involved gathering information for the circumstances reported for scald injuries in children, in a hospital setting. The retrospective database and chart review, presented in chapter 7, described the burn conditions reported for children with severe scald injuries which required grafting. Chiefly, this clinical review was conducted to evaluate how findings from the experimental animal studies, which form the bulk of work for this thesis, compare to scald injuries seen clinically in children. However, it is interesting to
consider whether an analysis of clinical observations alone could have provided the level of scientific evidence required to update scald injury prediction data.

For regulators setting safety standards for products and hot water use, precise threshold values for the water temperatures and durations of exposure involved are desirable. For the controlled and standardised animal experiments described in this research project, objective measurements for both the burn conditions and the resultant depth/severity of tissue injury were made. Unfortunately, in a clinical setting, objective measurements were rarely available and instead subjective estimates for the water temperature and duration of exposure were given. The precision of these estimates may be further compromised by the exactness and truthfulness of the recall of the circumstances surrounding the scald event by parents and caregivers at this stressful time. Fortunately, by means of an established and detailed pro forma (which is completed by clinical research staff at patient presentation), additional information such as whether the water was boiling, freshly boiled or whether milk had been added to beverages was available for many cases. Literature regarding cooling rates of hot beverages and kettles was then used to refine estimates for the water temperatures involved. However, it remained difficult to establish precise burn conditions for a severe scald injury from a spill/splash or immersion scald event.

The paediatric data presented in chapter 7 was retrospective, and inconsistencies in the quality of data collected was a substantial limitation. Disappointingly, one quarter of the cases of severe scalds (those treated with split-thickness skin grafting (SSG)) did not have adequate information available in either the database or patient medical charts to allow estimates for the burn conditions involved. It is interesting to reflect on whether an appropriately designed, prospective clinical observational study, could have provided sufficient evidence to replace the animal studies. Even with a prospective study, the small number of cases of severe scalds requiring SSG (47 cases over 4 year time period) and the wide variety of circumstances for each individual scald event would make quantitative statistical analysis challenging. Aside from variations in the situations surrounding the scald event e.g. clothed or not, first-aid given; additional variables such as the age of the child, the site of injury, the thickness of the skin, and concurrent disease conditions may affect the severity of injury and the healing potential of a burn. Therefore, although a prospective clinical observational study would contribute substantially to improving understanding of the burn conditions involved for severe scald injuries in children, in terms of evidence-based scald injury prediction data, in isolation, such a study would not be as robust as the
experimental animal studies presented here.

8.2.2 The porcine scald model developed successfully represents human scald injuries

By definition a 'model' is not a perfect replicate of the human situation. One of the key criteria considered when selecting the animal model for this research project, was that results obtained would be translatable to humans. Utilisation of an animal model provides a unique opportunity to study the complex pathophysiology of a burn injury from creation to healing, in a reproducible manner. For many decades pigs have been used as the standard model for wound healing (247). Not only is pig skin morphologically similar to human skin (firmly attached to underlying structures, sparse hair covering, comparable dermal-epidermal ratio and vascularity), but pigs also have similar wound healing characteristics as humans (wounds predominately re-epithelialise rather than contract for closure). Therefore, despite the expense and logistical issues associated with handling and housing large animals, a porcine model was chosen for this research.

8.2.2.1 Method for creating and evaluating reproducible scald injuries established

The first step in the development of the porcine model was to successfully establish a reliable and safe method of scald burn creation (chapter 3). To maintain a consistent water temperature over long durations, a novel scald burn device was used which refreshed the water. Over a 5–10 minute exposure time, the temperature of the water within the device was maintained at 49.8 ± 0.1°C when the test temperature was set at 50°C, indicating the system was efficient. With experience, additional methods for further reducing variation in the temperature of the water within the device (especially for shorter duration exposures) were identified, e.g. pre-warming of the scald device, insulating the inflow tubing, and setting the water bath to 3°C higher than the desired target temperature (for temperatures ≥ 70°C). For acceptability of the model, it was equally important to demonstrate that the risk of accidental burn injury to investigators or the animal was minimal. The unique design of the scald device allowing for excess water to be removed (vacuum suctioned out) prior to lifting the device from the skin, assisted in safeguarding against any unintentional injury. No unintentional injury to investigators occurred and with appropriate safety precautions a safe method of scald burn creation was established.

The created burns showed uniformity between replicates for burn appearance, confirming the regularity of contact between the heat source (water) and the skin. The wounds were
large enough for multiple, well-spaced, biopsies to be taken at different time points in the early post-burn period (7 days). This allowed for burn wound progression (over 7 days) to be evaluated, ensuring the full extent of damage to the dermis could be appreciated.

Demonstrating consistency in depth of tissue damage between replicates was essential to verify the reproducibility of the model. Therefore, the second critical stage in the development of the model was to identify markers for tissue injury using histological analysis and establish a standardised methodology for quantifying the extent of tissue injury. In conjunction with an experienced dermatohistopathologist and a review of the literature, a standardised approach to determining tissue injury in H & E stained sections was established and has now been peer-reviewed and published (223). Importantly, using this method a high intra-observer reliability rating \( r = 0.87 \) was achieved. Additionally, observations regarding the reliability of using vascular injury as a marker for the deepest extent of tissue injury are in agreement with Singer et al. (55), whose group have published extensively in this area (61, 149). Establishing this method was important, as histologically determined depth of tissue injury was one of the two main outcome measures for the experimental animal studies.

Finally, it was important to establish a reliable and clinically relevant approach for evaluating the time for burns to fully re-epithelialise. For human burn injuries, time to re-epithelialise is an important clinical indicator of burn severity. The end-point of 21 days is a well-accepted, modern criterion, used both clinically (5, 248) and experimentally (124) in burns research for assessing the healing potential of a burn. Therefore, for the experimental animal studies, time to re-epithelialise for the different burn conditions was investigated by studying burns for 21 days. Additionally, there is contention between research groups as to the effect that repeat biopsies may have on wound healing and subsequent analysis for re-epithelialisation at later time points. Visually, we observed substantial disruption to the wound in the area surrounding sites previously biopsied and data from LDI analysis confirmed disturbance to the wound extended beyond biopsy margins. To better replicate the healing circumstances of human burn injuries, where the whole burn area is available for re-epithelialisation assessments, burns which had multiple biopsies taken were not included in the study of re-epithelialisation outcome.

The study achieved its aim of developing a reliable, safe and consistent porcine model of scald injury (223). By enabling testing for a broad range of water temperatures and exposure
durations, the model reproduces the circumstances for human scald injuries, allowing for the mechanisms involved in scald creation and wound progression to be investigated. Successful development of this method was crucial as it formed the basis of all experimental work for this thesis.

8.2.2.2 Skin thickness of pigs was comparable to that of human children

Young children, in particular toddlers, have an increased incidence of scald injuries compared to adults (90, 91). Therefore, a porcine model with a skin thickness similar to that of a human child was desirable and is arguably of most translational relevance. Prior to commencement of this research project our group had extensive experience working with 12–14 week old pigs. As shown by the systematic review of porcine burn models (chapter 2), for burn and wound healing research, this age group of pigs are the most commonly used model (average 15.5 ± 8.8 weeks old). The average skin thickness (determined histologically) for the 12 week old pigs used for this research project was 2.09 ± 0.2mm, which is comparable to the average porcine skin thickness described by studies included in the systematic review of 2.27 ± 0.6mm (234). At the commencement of this research project, skin of this thickness was considered more similar to thickness values reported for human adults, rather than children (94, 98). However, a key problem with this comparison was that the skin thickness values in pigs were determined by ex vivo histological analysis, whereas the comparative data used for adults and children was established using in vivo measurement with ultrasound. One major drawback with this approach was the assumption that skin thickness measured in vivo was approximately equivalent to that measured ex vivo. It was not until part way through this project, when an ultrasound machine was procured (for the purpose of evaluating the placement of subdermal temperature probes), that we observed skin thickness measured histologically was substantially thicker than in vivo ultrasound measurements.

A unique study to determine the magnitude of change and the influence of excision and biopsy processing procedures on porcine skin thickness measurements was described in chapter 5. This study compared skin thickness measured in vivo using ultrasound and ex vivo using standard histological processing techniques. Notably, skin was significantly thicker (p < 0.0001) after histological analysis of routinely processed formalin and frozen sections than in vivo ultrasound determined measurements. It was proposed that the high content of elastin in the dermis causes the skin to recoil once skin tension is reduced with excision. These findings are not in isolation and similar results have been reported by others
for human (100), dog (225) and cat (226) skin. Nevertheless, there appears to be a lack of awareness of this concept in the broader skin research community. Therefore, it was important to publish these findings (230) and alert researchers to the possibility of overestimation of depth/thickness with biopsy samples as a result of processing artefact.

In the overall context of this thesis, the findings from the skin thickness study ((chapter 5, (230)) were crucial to the progression of the animal studies and helped to verify the appropriateness of using 12 week old pigs to model scald injuries for human children. The skin thickness of the 12 week old (25–27kg) pigs used for the main experimental studies presented in this thesis was 1.42 ± 0.23mm (determined using ultrasound). This is similar to skin thickness measurements (determined using ultrasound) reported for human children aged 2–13 years, which range in thickness (depending on anatomical location) from 0.89 ± 0.14mm to 1.7 ± 0.41mm for skin from the elbow crease and the interscapular region, respectively (94).

8.2.3 Contemporary estimates for the thermal properties of in vivo skin

In chapter 4, the thermal properties of skin were examined by using a mathematical model, to interpret the subdermal temperature data collected from the experimental animal studies presented in this thesis (189). This modelling study was unique, as the experimental data set presented is the first in vivo study to directly compare heat conduction in skin of different thickness subject to the same burn conditions. For long duration, moderate temperature water, estimates of thermal diffusivity (α) appear to be independent of the burn duration, burn temperature and skin thickness. Given the modest temperature range of the experimental scenarios considered (50–60°C) this result is not unexpected. In contrast, estimates of the thermal loss rate (k) were more variable and a larger loss rate was reported for the longer duration burn conditions. For these longer duration exposures (≥ 300 seconds) it is likely the rate of heat loss to the blood supply was increased. While it remains difficult to speculate, it is suggested that in response to heat exposure, vessels in the local tissue area may dilate, enhancing blood perfusion and increasing heat dissipation. Overall, estimates were: \( \alpha = 0.03 \text{mm}^2/\text{s} \), and \( k = 0.002 \pm 0.002/\text{s} \). This average estimate for \( \alpha \) is lower than previously reported in vivo estimates from a study by Henriques (194). Key methodological differences between studies likely contributed to this disparity in results. The contemporary estimates provided here have application not only to thermal injury prevention studies, but also more broadly to heat transfer modelling investigations e.g. forensic
medicine and thermal therapy studies. Additionally, in the context of this research project, it was important to develop our own estimates for the thermal properties of skin using data obtained from the scalding experiments. In the future, these estimates could then be directly applied to further modelling investigations exploring the relationship between heat conduction and tissue damage for scald injuries.

8.2.4 Updating the evidence for the water temperatures and durations of exposure for severe scald injuries

8.2.4.1 Scald injury prediction data from the 1940s overestimates injury severity

This thesis clearly demonstrates that existing predictions for deep scald injuries, reported in the 1940s, overestimate the expected burn severity. Strong evidence is presented to support these findings using comparable animal studies (chapter 6) and paediatric data (chapter 7).

In chapter 6, the updated time and temperature threshold data provided by the animal studies was directly compared to estimates established by Moritz and Henriques in the 1940s (1). Using histological evaluation of depth of damage to the dermis and clinical observations of time to re-epithelialise, results from this study demonstrated that Moritz and Henriques data overestimates injury severity for both spill/splash and immersion scalds. Their data gives a false impression that an accidental spill/splash (short duration exposure) to water at 60–65°C will cause deep burns. Here, the data clearly shows that this is not the case, and a higher threshold temperature of 85°C is more likely to result in a severe burn. This higher exposure requirement is also supported by recent numerical modelling work of Abraham et al. (79), who reported 82°C as the benchmark temperature for a major burn from a spill incident. Most importantly, findings from the animal studies are further validated by the clinical paediatric burn injury data (chapter 7). The paediatric data study found no evidence to support Moritz and Henriques findings that a short exposure to 60–80°C water would result in a severe scald injury. On the contrary, the circumstances reported for severe paediatric scald injuries (requiring grafting) suggest that substantially hotter temperatures (≥80°C) are implicated.

In Australia, poor compliance to hot tap water safety regulations is reported (232, 233). Anecdotally, it is observed that water temperatures well in excess of 50°C are delivered to sanitary fixtures (e.g. bathroom taps) in many homes. Here, evidence provided by the animal studies (chapter 6, (237)) shows that for water less than 70°C, long exposure times (greater than 15 seconds) are required to result in a severe immersion scald injury. Not surprisingly,
these findings are also in disagreement with Moritz and Henriques data, which reported substantially shorter exposure times (e.g. exposure for 1 second to 70°C water) (1). This disparity is notable, as the data from Moritz and Henriques studies is used not only to inform burn preventions strategies, but also legally cited for cases where an inflicted mechanism of scald injury is suspected. Although inflicted scalds represent only a small percentage of paediatric scald injury cases (19, 249), the consequences of using erroneous injury prediction estimates are considerable. Unfortunately, child abuse by inflicted hot tap water immersion remains a concerning issue (19). Clinicians may be asked to provide an ‘expert opinion’ to police or to court proceedings regarding the circumstances of a scald injury. In the absence of contemporary data for human skin, it is recommended the updated scald injury prediction data provided by the porcine studies described in this thesis (Table 8-1), replace previously accepted estimates from the porcine studies of the 1940s.

Table 8-1 Temperature and duration of exposure for a clinically relevant DDPT to FT scald injury which will not fully re-epithelialise within 21 days

<table>
<thead>
<tr>
<th>Water Temperature</th>
<th>Duration of exposure</th>
</tr>
</thead>
<tbody>
<tr>
<td>50°C</td>
<td>&gt; 10 minutes</td>
</tr>
<tr>
<td>55°C</td>
<td>2 ≥ 5 minutes</td>
</tr>
<tr>
<td>60°C</td>
<td>30 ≥ 60 seconds</td>
</tr>
<tr>
<td>70°C</td>
<td>&gt; 15 seconds</td>
</tr>
<tr>
<td>85°C and 90°C</td>
<td>5 seconds</td>
</tr>
</tbody>
</table>

8.2.4.2 DDPT burns which will not fully re-epithelialise by 3 weeks display extensive damage to the dermis (≥ 75% depth of dermis damaged)

Utilising vascular damage as a marker of tissue injury, the porcine studies reported in this thesis proposed an injury to the dermis of 75% or deeper would result in a clinically relevant DDPT burn. Although there are few similar studies with which to compare this threshold, this percentage is slightly higher than suggested for contact burns (124, 146). More commonly, a SPT burn is only distinguished from a DDPT burn by simply reporting damage extending to the ‘deeper portion’ of the dermis (39, 46). Additionally, where quantitative results for percentage depth of dermis damaged are reported, it is not always apparent how the level of tissue damage observed relates to the healing potential of a burn. Here, for 61 individual burns, the association between re-epithelialisation outcome and histologic depth of damage was evaluated for 10 different burn conditions. This provided good evidence to suggest that
applying a threshold of 50% depth of damage to the dermis to classify a clinically relevant DDPT injury overestimates burn severity. This finding is notable as histology remains the standard criteria for burn depth evaluations in a research setting.

8.2.4.3 LDI scans at day 3 post-burn do not reliably predict the healing outcome of DDPT scalds

Laser Doppler Imaging is promoted as a clinical diagnostic tool for distinguishing between SPT and DDPT burns. Many human studies report good reliability of LDI scans at 2–4 days post-burn for predicting the healing outcome of moderate to severe burns (121, 132, 236). However, this high level of reliability was not replicated in the porcine studies presented in this thesis ((chapter 6, (237)). Here, although LDI scans from 24 hours post-burn were observed to reliably measure the hyperemic response of superficial dermal injuries, they were less able to differentiate deeper dermal injuries. For example, at day 3, perfusion values for moderate severity burns which fully re-epithelialised by day 21 were not significantly higher than for burns which did not fully re-epithelialise. Similarly disappointing results for distinguishing deep partial thickness injuries using LDI in a porcine burn model have been reported by Fourman et al. (127). They suggested that the poor penetrating ability of LDI (up to 1 mm) may explain difficulties in interpreting intermediate and low LDI perfusion measurements. However, evidence presented in this thesis ((chapters 5 and 6, (230, 237)) demonstrates that pigs used for this study had skin similar in thickness to that of humans (determined by ultrasound). Alternatively, it should be considered that in the porcine studies LDI scans were performed whilst the animals were under a general anaesthetic. Clinically, most LDI scans of children and adults are conducted while the patient is awake or mildly sedated. While the anaesthetic used here was essential to ensured pigs were immobilised for dressing changes and LDI scans, anaesthetics are known to alter the vasoactivity of the skin (250, 251). Perhaps this factor, combined with the slightly different distribution of blood vessels in pig skin compared to human skin, may have affected the reliability of LDI for distinguishing DDPT scald burns. Notably, in the porcine studies, repeat LDI measurements were shown to better demonstrate changes in wound perfusion and increase the strength of healing predictions.

8.2.4.4 Repeat biopsies affect reliability of LDI perfusion results and may have an influence on wound healing

Unique to this study, a large number of LDI scans from equivalent burns with and without biopsies were available for comparison. While biopsy sites were excluded from perfusion
calculations, the size of this region was variable and the increased perfusion areas surrounding the biopsy sites were not excluded. Perfusion levels in biopsied burns showed increased variation between replicates and were significantly higher than burns with no biopsies (statistically significant at day 7 for all spill/splash burns except 75°C for 5 second scalds, \( p < 0.03 \)). This indicates that LDI results obtained from biopsied burns are less reliable and likely to overestimate perfusion. Therefore, for burns which had biopsies for histological analysis in the early post-burn period (7 days), it was not possible to compare histologically determined extent of vascular injury and wound perfusion results. In a broader context, for burns researchers, where wounds have previously been biopsied, caution is recommended when interpreting perfusion results from LDI or other diagnostic modalities. Additionally, while not investigated here, it is of interest to consider whether the increased perfusion seen in burn wounds with repeat disturbance (biopsies) may actually indicate that biopsies increase the rate at which these wounds re-epithelialise. To reduce scarring, early interventions which accelerate re-epithelialisation are desirable (252). Further investigations in this area could consider whether interventions which create minor disruption to the wound early post-burn, such as medical needling therapy or fractional CO\(_2\) laser ablation, have the potential to enhance re-epithelisation rates of moderate to severe burns.

8.2.4.5 Majority of severe scalds in Queensland children result from an accidental spill or splash with water from a recently made hot beverage or recently boiled kettle/saucepan

The retrospective database review for severe paediatric scald injuries presented in this thesis (chapter 7) demonstrated that the majority of severe scald injuries (which required grafting) resulted from an accidental short exposure to water from a recently made hot beverage or recently boiled kettle or saucepan. With the cooling rates for hot beverages and kettles known, it is possible to estimate the likely water temperatures involved. Evidence provided by this study shows that severe scald injuries (requiring grafting) in children do not commonly involve temperatures below 80–85°C. These findings are similar to those from the experimental animals studies presented in chapter 6 (237) and further demonstrate the inaccuracy of the data for deep scald injuries presented by studies from the 1940s (1). This study of paediatric data achieved its aim of improving understanding regarding the severity of, and the mechanisms by which, scald injuries occur. Importantly, findings from the study of scald injuries in children (chapter 7) were similar to those from the porcine scald model (presented in chapter 6). Overall, for this research project, the validity of the
recommendations from the experimental animal studies was strengthened by the inclusion of this paediatric burn data.

8.3 Strengths

The experimental porcine studies presented in this research project deliver one of the most comprehensive data sets reported for thermal injury investigations of this nature. For this study, using 26 pigs, 20 different burn conditions were investigated and over 200 individual scald burns were created for evaluation. This provided an extensive data set for analysis, which is considerably larger than reported for comparable studies. The systematic review of porcine burn models (chapter 2, (234)) showed most burn injury studies are conducted using only a few animals, with an average of six pigs per study. Additionally, it is common for only a few burn conditions to be tested. Here, although the sample size for each burn condition was restricted, overall a large number of burns were created. The large number of burns allowed grouped analysis for healing predictions using both LDI perfusion results and re-epithelialisation outcome. These analyses provided valuable information and increased the power of the statistical examinations.

A key strength to the methodological approach of the experimental investigations was the comprehensiveness with which tissue injury severity was assessed. A variety of methods were used to determine burn depth for multiple time points over the early post-burn period (7 days). In contrast to the studies of Moritz and Henriques (1), here quantitative results are presented for histologically determined depth of damage to the dermis. Importantly, sequential biopsies for the same burn ensured the full extent of tissue damage was appreciated. In addition to histology, the wound healing potential of the burns was evaluated using Laser Doppler Imaging to measure blood flow to the burn. Lastly, for a subset of burns, injury severity was also evaluated by considering time to re-epithelialise. This outcome measurement is arguably the most relevant clinical indicator for a severe burn injury. In a clinical setting, burns considered unlikely to fully re-epithelialise by 3 weeks are often managed surgically. Therefore, the translatability of results from this study is strengthened by using this clinically significant outcome measure.

Recommendations informed from the experimental studies are also strengthened by considering their application in a clinical setting. For obvious ethical reasons it was not possible to validate findings from the experimental animal model by conducting testing in
humans. Therefore, to support interpretation of results to the situation for a human child, a separate study was conducted to investigate the burn conditions reported for severe paediatric scald injuries. Evidence from this paediatric database review supports the scald injury prediction data provided by the experimental investigations, enhancing the translational relevance of this research.

8.4 Research limitations

8.4.1 Limitations of using an experimental animal model for translation to human scald injury events

Despite the measures taken to improve the translational relevance of this research study (discussed above) the generalisation of findings from the porcine scald model to the situation in humans remains a major limitation. Regardless of how well-designed a model may be, porcine skin is not human skin, therefore extrapolation of results to humans requires careful interpretation. Additionally, by necessity, the model developed represents a simplification of the conditions for a human scald injury event. In a real-life setting, the circumstances for each individual scald event are highly variable. For this study it was not feasible to investigate every possible time and temperature combination, and additional variables such as first aid treatment and the presence of clothing were not considered.

The time and temperature relationships described by this study were established under controlled and standardised experimental conditions. The impact this may have on resultant tissue injury severity and wound healing is unknown. Clinically, it is recognised that re-epithelialisation rates may be influenced by a variety of factors, including pain and anxiety (253). For this study, while procedures for dressing changes, wound cleaning and pain relief were comparable to the treatment given to children with scald injuries, dissimilarities remain. Unlike the situation in humans, for this study, pigs were under a general anaesthetic during scald creation and for dressing changes. Additionally, pre-emptive pain relief was given and strong opioid analgesics were used. It is possible that the healing capacity of the pigs, treated under these experimental conditions, may be enhanced from that of a child. Under those circumstances, the thresholds presented here for severe scalds (not fully re-epithelialised by 3 weeks) would be more likely to underestimate injury severity.

8.4.2 Burn conditions comprehensively tested for only one skin thickness

At the commencement of this research project it was anticipated the animal studies would
involve both 12 week old (25–27kg) and 5 week old (8–10kg) pigs. This approach was designed based on the assumption that the skin thickness of 12 week old (25kg) pigs was a better approximation for human adult skin thickness rather than a child’s skin thickness. Given the groups previous experience with 12 week old (25kg) pigs, it was decided these animals would be utilised to establish the scald model and conduct preliminary testing. Following this, testing for a broad range of burn conditions in the younger (5 week old) pigs was proposed. Indeed, a small pilot study using a scaled down version of the scalding device was conducted with four, 5 week old pigs. This pilot study provided valuable data which was used to inform the mathematical modelling presented in chapter 4 (189). However, it was difficult to obtain ongoing ethical approval for continuing investigations with 5 week old pigs. Additionally, replication of all burn conditions in the thinner skinned (5 week old) animals was both cost and time prohibitive. Therefore, testing for all burn conditions was limited to the 12 week old pigs.

The skin thickness of the 12 week old pigs was ultimately determined to be more similar to that of a human child than previously assumed. However, the skin thickness of the 5 week old pigs was not determined using ultrasound as the ultrasound machine had not been acquired when testing was done in these animals. This limited comparisons for in vivo skin thickness between the 5 week old pigs and both the 12 week old pigs and human children. The overall scope of the experimental studies was limited by only comprehensively testing burn conditions in one age group of animals, with similar skin thickness. Future application of the scald injury severity data to mathematical modelling would benefit from having additional data for different skin thicknesses. In future, dependant on ethics approval, testing of the threshold conditions identified in the 12 week old pigs for DDPT burns could be considered in younger pigs with thinner skin.

8.4.3 The number of replicates for each burn condition was limited

Even with an experienced team of investigators and a standardised method of burn creation, variation between replicates is to be expected (180, 194). Indeed, one of the major challenges with in vivo experimental studies is to minimise animal use, while still obtaining robust data for statistical analysis. Here, given the wide range of burn conditions tested, it was not ethically or logistically feasible to have an overly large number of replicates of each burn condition. A power calculation to determine the minimum number of replicates required was performed using data from the first four animals tested. However, variation in depth of
damage determined histologically between replicates was greater than anticipated for some burn conditions, limiting the power of statistical analysis.

The average standard deviation in histologic depth of damage between replicates was 13–19%, which is similar to variations observed by others (180, 194). However, variation between replicates was greater for the spill/splash scalds (5 second duration) than the immersion scalds. Differences were greatest at day 3 for the high temperature (≥ 75°C) spill/splash scalds, where the standard deviation for histologic depth of damage ranged from 27–39%. It is possible batch variation contributed to differences seen. As the experimental studies progressed, experience with the scalding device increased. Refinements were made to improve the consistency of the delivered water temperature for high temperature scalds (e.g. pre-warming the device, setting the water bath to a higher temperature than the test temperature). It is also suggested, that with longer duration immersion scalds, any relative errors in exposure time and their influence on burn depth are minimised (180). Additionally, although the animals used were of a similar age and weight, biological variation between animals for susceptibility to thermal injury is likely. Notably, although burn conditions were randomised for anatomical location (sites A–D), complete randomisation of the burn conditions across all animals did not occur. Substantial time was required to reset, adjust and stabilise the water temperature for different burn conditions. Therefore, for each individual animal the water temperatures tested were within a similar range, in this manner temperature adjustments between burns was reduced and the length of the procedure was minimised. Consequently, it is probable both methodological and biological variations contributed to the differences seen between replicates for depth of tissue injury. Improvements to future study design may consider the feasibility of creating a larger number of burns on each animal (e.g. six on each side instead of four) and increasing the number of replicates (particularly for short duration exposures), without increasing animal numbers.

8.4.4 Subdermal temperature probes were placed deeper than desired

The distance between the bottom of the dermis and the tip of the subdermal temperature probes was greater than anticipated. The subdermal temperature probes were determined by ultrasound to be 2.6 ± 0.59mm from the bottom of the dermis. Placement of the temperature probes closer to the base of the dermis would have improved the strength of the modelling presented for heat conduction in skin ((chapter 4, (189)). Additionally, the relationship between change in subdermal temperature and tissue damage may have been
better appreciated. For this study, the insertion point for the probes was close to the outer margin of the burned area and this dictated the angle of advancement of the inflexible metal stylet. For future studies, inserting the probe further away from the burn site would allow for a shallower angle of advancement, which may result in the tip of the probe being closer to the bottom of the dermis.

Placement of the temperature probes into the dermis itself was considered to allow for a greater understanding of how heat is transferred through the different layers of the skin. However, inserting a 0.8mm diameter probe into tissue which is only 2mm thick was logistically problematic. Additionally, it is unknown how results for tissue damage may be affected by inserting probes within the dermis, which would compromise the integrity of the tissue structure. The placement of the subdermal temperature probes deep within the subcutaneous tissue limited analysis for change in temperature of different layers of the skin. Future experiments may include investigating the use of different apparatus to take multiple temperature measurements at different depths.

8.4.5 Direct comparison between scald and contact burns was not possible

It remains difficult to speculate on whether differences described clinically for contact and scald injuries are due to altered pathophysiology or to disparities for intensity of the heat and duration of exposure. Although findings from the systematic review of porcine burn models (chapter 2, (234)) strongly indicate that mechanism of injury has an effect on the severity of a burn, experimental evidence for this lacking. Depth of dermal injury from the experimental scald injuries (chapter 6, (237)) was observed to be greater than that reported in the literature for contact burns created using the same temperature and duration of exposure (55). However, the actual amount of heat delivered to the tissue by these different mechanisms is not equivalent. Substantial differences in the thermal properties of water compared to different contact burn materials e.g. metal, glass and ceramics, limited direct comparison between scald and contact burns.

8.5 Clinical implications and recommendations

8.5.1 Compliance with existing hot tap water safety regulations is essential

In Australia, hot tap water safety legislation mandates installation of tempering valves to ensure water delivered to sanitary fixtures does not exceed 50°C for domestic dwellings and 45°C for nursing homes and child care centres (214). In the porcine model (chapter 6,
(237)), at day 3 post-burn, a significant relationship between increasing duration of exposure and increasing depth of dermal damage was observed for 50, 55 and 60°C water scalds (p ≤ 0.005). Additionally, for equivalent durations of exposure, water at 55°C caused significantly deeper dermal damage than 50°C (p < 0.05). These findings highlight the importance of compliance with existing hot tap water safety regulations as just a 5°C increase in temperature (from 50 to 55°C) contributes significantly to increasing the risk of sustaining a severe burn.

Similarly, the paediatric scald injury data (chapter 7) shows that no tempering valves were present in the homes of all cases of severe scald injury from immersion in hot tap water (n = 4). In one instance, a duration of exposure of 1–2 minutes in hot tap water was described. For this case, if tempering valves had been installed and the water was ≤ 50°C, evidence from the porcine model shows this duration of exposure would not result in the child sustaining a severe burn. Indeed, at day 3 in the porcine model, 50°C for 2 minutes showed little visible signs of burn injury and no histologic evidence of damage to the dermis (237). A recommendation from this research project is to increase awareness regarding the danger posed by water in excess of 50°C from hot taps. In conjunction with Kidsafe Australia (Queensland), data from this thesis will be used to develop and promote updated scald injury prevention material. An additional approach to improving compliance with, and maintenance of tempering valves, could include introduction of policy to enforce mandatory inspection for compliant tempering valves prior to leasing or selling a property (similar to the swimming pool safety laws).

8.5.2 Updated scald injury prediction data has been used to inform medicolegal proceedings

For cases where an inflicted mechanism of scald injury is suspected, the consequences of relying on erroneous injury prediction estimates to inform judgements are considerable. The time and temperature thresholds for severe scalds, established by the animal studies presented in chapter 6, represent the ‘worst case’ scenario for a human child exposed to similar burn conditions. This data provides valuable scientific evidence which, in conjunction with clinical observations, can be used to support judgements regarding whether or not a burn may have been accidental or inflicted. To date, on four occasions in 2017, findings from this thesis have been used by clinicians at the Lady Cilento Children’s Hospital to provide evidence to police regarding the circumstances surrounding immersion scalds where an
inflicted mechanism of injury was alleged. Below is an excerpt from a statement provided to police for one of these investigations:

‘According to police investigation, we have been informed that the hot water that came from the hot tap in the bath where X sustained their injury had a maximum temperature of 60ºC. We know from our own research, it would take at least 30 seconds of contact time with water of this temperature to sustain the depth of burn which we have identified. This is a conservative estimate and the duration of contact could have been significantly longer with a contact time of up to two minutes.’

It is anticipated the published data from this work may also be cited in legal proceedings, replacing the outdated injury prediction estimates from the 1940s (1).

8.5.3 Scientific evidence is provided for regulators setting safety standards

Hot beverage scalds are the leading cause of burn injury in Queensland children (17). To reduce the risk to a child of sustaining a severe scald injury, research from this thesis recommends that the serving temperature of hot beverages be below 85ºC. Whilst hot beverages are frequently served at temperatures between 71 and 85ºC (254), industry standards for beverage temperatures are not clear, with hospitality industry literature recommending holding temperatures in excess of 85ºC (255). While it may be more difficult to control the serving temperature of hot beverages in a home setting, in a commercial setting this is feasible as the brewing and holding temperatures of coffee machines and ‘boiling water taps’ can be measured and adjusted. Findings from this thesis can be used to inform regulators developing industry standards for hot beverage temperatures and provides updated information for burn prevention programs targeting hot beverage scalds.

This research has also been used to provide scientific evidence for regulators setting safety standards in Australia for oven door temperatures. In the absence of comprehensive contemporary burn injury prediction data for a broad range of conditions, findings from the experimental scald injury studies were used to support recommendations to reduce the allowable oven door temperatures for gas cookers. In future, as suggested by the systematic review of porcine models ((chapter 2, (234)), it would be beneficial to use modelling to estimate the equivalent thermal dose for different materials which can cause burn injury. This data would further strengthen the reliability of recommendations for future product safety considerations.
8.5.4 Recommendations for using LDI to assess the healing potential of a burn

The findings presented in chapter 6, regarding the reliability of LDI for predicting the healing potential of burns, highlights the need for caution when interpreting intermediate and low perfusion measurements for scald burns at day 3 post-burn. Therefore, where feasible, it is recommended to repeat LDI scanning for these burns at a later time point. This recommendation is applicable in a clinical setting where perfusion results from LDI scans are used to guide decisions regarding the surgical management of a burn. Findings from this research suggest that at day 3, low perfusion may be observed for some burns that will ultimately fully re-epithelialise by 3 weeks. Under these circumstances, repeating the LDI scan would improve the strength of LDI healing predictions, thereby possibly avoiding unnecessary grafting of these burns.

8.6 Future directions and conclusions

The work conducted for this thesis has prompted several questions concerning the pathophysiology of burn wounds and the consideration of future work in the following areas:

- A public education campaign to promote recommendations for preventing severe scald injuries in children, particularly those for domestic hot tap water safety and the serving temperature of hot beverages.
- Develop clear, evidence-based clinical guidelines to assist clinicians with providing medicolegal reports where inflicted scald injuries are alleged.
- Using the time and temperature data established by this research as a guide, further experiments comparing tissue injury severity are recommended, examining the possible effect of:
  - appropriate first-aid treatment with running water
  - presence of clothing, with different materials/fabrics
  - differences in skin thickness
- An experiment to examine the temperature change at different depths within the dermis when a specific heat dose is applied.
- Further mathematical modelling to examine how heat conduction in living skin relates to tissue damage.
- Further mathematical modelling to estimate the equivalent thermal dose delivered for different materials, particularly for contact burns.
- Experiments to further examine possible differences in the pathophysiology between scald and contact burns.

The work presented in this thesis comprehensively re-examines the relationship between burn temperature, duration of exposure and tissue injury severity for scald burns. Strong scientific evidence has been obtained for the burn conditions (temperature and duration of exposure) for a clinically relevant severe burn from a spill/splash or immersion scald event. This research provides valuable evidence-based scald injury prediction data. This data can now be publicised widely to update global burn injury prevention strategies and provides an important scientific resource for clinicians considering cases where an inflicted mechanism of scald injury is suspected.
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Appendices
Appendix A

Optimising histopathological staining methodology for assessing cell viability and tissue damage

Overview

As explained in the literature review of this thesis (chapter 1), there are a wide variety of different histological staining techniques available to identify cell and tissue damage in burned skin. This experimental study describes and discusses some of the additional histopathological staining methods evaluated for this research project.

The timing and mechanisms behind the cellular events of burn wound progression are complex and poorly understood. This information is essential for further development of early intervention therapies designed to limit burn injury progression. The multitude of tissue samples collected during the creation of the porcine scald burn model ((chapter 3, (1)) and subsequent experiments ((chapter 6, (2)) enabled exploration of the cellular biology of burn wounds and their progression. In this study, different histopathological staining approaches were investigated and their reliability for assessing cell viability and structural tissue damage in the acute post-burn period (7 days) and for wound healing (up to day 21) was observed.

This study was not included in the main section of this thesis as the staining techniques used proved to be unreliable and inconsistent. Although these staining methods were not ultimately used to describe tissue injury severity in any of the published works from this thesis, it remains important to report on the strengths and weaknesses observed for these approaches.

This study is not based on any published works.

Introduction

The depth of a burn wound is not static. The dynamic phenomenon of burn wound progression is of particular importance for partial thickness burns where an initial superficial or intermediate severity partial thickness burn may progress over a period of 2–4 days to a deep partial or full thickness burn (3). Clinically, burn wound progression is relevant for two main reasons. Firstly, it confounds early classification of tissue injury severity, making diagnosis of burn injury depth more challenging (4). Secondly, the depth of a burn is a
significant determinant of morbidity and treatment (4, 5). With increased depth of damage to the dermis, a patient is more likely to require extensive treatment, hospitalisation, and surgical intervention which places them at increased risk of developing hypertrophic scarring. However, the timing and mechanisms behind the cellular events of burn wound progression are still poorly understood (3-7).

An important consideration for researchers is the challenge of differentiating between structural and functional cell damage early post-burn. Injured cells which survive the initial thermal insult may either recover or progress to become non-viable, causing a deepening of the burn wound over time. One of the disadvantages of routine histochemical stains, such as Haematoxylin and Eosin (H & E), is the inability to differentiate between cells which appear morphologically intact but are actually injured to the extent that they are metabolically inactive (8-10). Improving differentiation between cells which are functionally viable or inactive in the immediate post-burn period would increase accuracy of classification for tissue injury severity and may also help to clarify the role of burn wound progression.

Tetrazolium-based colorimetric assays (MTT assay) are widely used to access cell viability in vitro in cultured cells. Viable cells which have active mitochondria can reduce the soluble, yellow tetrazolium salt ([3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide]- MTT) to an insoluble dark blue formazan crystal (10-12). Whilst this technique is used extensively in vitro it is less widely described in vivo, with the majority of the literature originating from studies investigating myocardial and cerebral ischemia (infarcts) (9, 13-16). Converse et al. (8) first described the use of a tetrazolium technique (neotetrazolium chloride stain) for assessing thermal injury in skin in 1965 for a rat contact burn model, reporting some success. However, it is not until the studies of co-workers Henze et al. (17) and Radke et al. (12) in the 1990s, that the use of MTT staining of fresh biopsy samples was investigated for assessing cutaneous thermal injury. Using a porcine scald burn model they incubated freshly obtained biopsy samples with MTT solution and observed microscopically a clear demarcation line between unstained (non-viable) and stained (living) tissue. Whilst these results are encouraging, both groups only investigated the MTT staining at one burn condition (75°C water for 25 seconds) which created a severe deep dermal burn. There is no other literature available which examines its use in more moderate temperature thermal injury and there is a paucity of studies regarding live tissue MTT staining for burn depth assessment despite its potential to rapidly assess cell viability.

The ability of immunohistochemical (IHC) staining to facilitate rapid identification of specific
cell types and tissue structures in the damaged skin tissue can also be used to determine depth of thermal injury and gain insight into the pathophysiologic mechanisms of burn wound progression (18-22). Recent work by Hirth et al. (18, 19) confirms the importance of careful interpretation of histologic appearance in the early post-burn period, proposing that histologic appearance of cell death can be delayed (by hours or even days). The authors recommend the use of staining methods which can rapidly identify markers of injury that are followed by loss of cell viability. One such suggested method is IHC staining for detecting apoptosis (18-22). The role of apoptosis (an organised process of programmed cell death) in burn wound progression is of particular interest. It is recognised that improved understanding of the timing and relative importance of this process may enhance development of therapeutics designed to block this mechanism of cell death and limit burn wound progression (6). Work in this area has been undertaken using a porcine contact burn model, however, to date there are no published studies using these techniques in a porcine scald model.

An alteration in collagen morphology is also commonly used as an injury marker for assessing burn depth (23-25). The pioneering work of Chvapil et al. (26) using a Masson’s trichrome stain describes a change in the colour of collagen when affected by heat injury. This change is attributed to differences in collagen fibre density and loss of the parallel alignment of collagen fibres. It is suggested that a change to the structure and arrangement of collagen in healing wounds may contribute to scar formation (27). This is important, as clinically a link between the healing time of scald wounds and hypertrophic scar (HTS) formation has been established (28). However, the exact mechanisms underpinning HTS formation are poorly understood. Therefore, it is important to evaluate changes in collagen morphology for moderate and severe burn injuries in the acute burn period and during wound healing.

The aim of this study was to examine different histopathological techniques described in wound literature to evaluate their performance in relation to identifying tissue injury, burn wound progression and wound healing, specifically for scald burns. Scald burns of different severity were created in an experimental porcine model (1) and full thickness biopsies were collected at 1 hour, and days 1, 3, 7 and 21 post-burn. Three staining techniques were examined: 1) modified 3-(4,5-dimethylthiazol-2-yl)-2,5-di-phenyltetrazo-lium-bromide (MTT) staining of fresh biopsy samples to assess cell viability; 2) IHC staining using Apoptag® to detect apoptosis; and 3) Sirius Red staining to visualise collagen morphology in burned
Methods and Materials

Animal Surgery and Burn Creation

All porcine tissue samples analysed during this study were collected as part of the main experimental study for this research project presented in chapters 3 and 6 (1, 2). All animal work was approved by the University of Queensland Animal Ethics Committee (Approval numbers: QCMRI/RCH/326/12/QCMRI/NHMRC and QCMRI/446/15/QCHF). All procedures were performed under a general anaesthetic and all animals were treated in a humane manner. Large White juvenile pigs of 25-27kg (approximately 12 weeks old) and 10kg (approximately 5 weeks old) were used for the study.

Method of general anaesthesia, scald burn creation and wound management was exactly as described previously in this thesis (chapter 3 and 6 (1, 2)). The only modifications to methods were changes to the tissue sample collection technique to allow MTT staining of fresh biopsy samples (outlined below). All other investigations in this study were performed on tissue that was previously collected and processed as part of the main experimental work presented in this thesis.

Tissue Sample Collection

Full thickness 8mm punch biopsies were collected from all burns and normal skin at 1 and 24 hours, and 3, 7 and 21 days post-burn. To minimise any local inflammatory effects post-biopsy, sequential biopsy sites were taken from representative areas that were well spaced (1). Each biopsy sample was divided into sections which were processed for histology in two different ways: 1) 10% formalin for 24–36hrs or 2) snap frozen in Cryo-Embedding Compound (Pelco®), then stored at -80°C. For a subset of samples, additional sectioning was performed for MTT staining.

MTT staining of fresh tissue biopsies to detect cell viability

Freshly obtained skin samples were stained with MTT solution using a modification of the technique described by Radke et al. (12). Tissue samples (1–2mm thick) were incubated in cell culture medium (DMEM, phenol red free Gibco/Invitrogen, Mulgrave, Vic, Australia) with 0.5, 1, 2 or 2.5 mg/ml MTT solution (Thiazolyl Blue Tetrazolium Bromide, Sigma-Aldrich, Castle Hill, NSW, Australia) for 1, 2, 4 or 24 hours at 37°C. After incubation, the samples
were washed three times in lactated ringer’s solution, then snap frozen in Cryo-Embedding compound and stored at -80°C. Stained samples were cut into 10µm thick sections with a Cryostat (Bright Instruments Co Ltd, Cambridgeshire, England). Images of stained sections were captured with a Nikon EP600 microscope (Nikon Instruments Inc, Melville, NY, USA) fitted with a Spot RT slider cooled CCD camera (SPOT Imaging Solutions™, Sterling Heights, USA). Viable (metabolically active) cells reduce MTT to a formazan dye in their mitochondria (9). Therefore, positive blue-to-purple staining of cells indicates that they are still viable, whereas inactive/injured cells remain colourless.

**Immunohistochemical staining to detect apoptosis**

All staining was performed on formalin-fixed, paraffin embedded, 5 µm thick sections. Cellular apoptosis was detected using deoxynucleotide transferase-mediated dUTP nick-end labelling (TUNEL) with the ApopTag® Peroxidase In Situ Apoptosis Detection Kit (Millipore, S7100, Merck Group, Darmstadt, Germany). Human tonsillar tissue was stained as a positive control and a negative control processed by omitting terminal deoxynucleotidyl transferase (TdT enzyme) from the labelling mix. Staining was performed using the ApopTag® kit recommended protocol, however, to optimise the technique for porcine skin, two of the steps were altered: the proteinase K incubation time was reduced from 15 minutes to 1, 5 or 10 minutes, and a more stringent wash using saline-sodium citrate (SSC buffer) was utilised instead of the standard stop wash buffer. The majority of images were captured with the Nikon EP600 microscope (described in the previous section). However, a small number of sections were digitally scanned using an Aperio slide scanner and images were captured using Aperio ImageScope v12 (Leica Microsystems PTY LTD, North Ryde, NSW, Australia). Similar to reported by others (29), positive brown staining/labelling of cells represented apoptosis where morphological changes were also present including: marked condensation of chromatin and cytoplasm (apoptotic cells), and cytoplasmic fragments with or without condensed chromatin (apoptotic bodies).

**Sirius Red staining to visualise collagen morphology**

A small subset of sections from all time points (1 hour and days 1, 3, 7 and 21) were stained with Sirius Red using a previously described method (27, 30). Formalin-fixed, paraffin embedded, 7 µm thick sections were dewaxed in xylene, and then washed though a gradient of ethanol to water. Sections were incubated in 0.1% Sirius Red F3BA (BDH Laboratory Supplies, Dorset, UK) in saturated picric acid for 1 hour at room temperature. After washing in water, they were placed in 0.1N HCl for 2 minutes and then after another rinse in water
the sections were dehydrated through ethanol and xylene before being permanently mounted. Due to the arrangement of its fibres, collagen has a natural birefringence which is heightened by Sirius Red staining. Under polarised light, the colour displayed is a result of the thickness of the fibre as well as the arrangement of the collagen molecules (27). In normal skin, thick collagen fibres with densely packed fibrils show a bright red to yellow colour. A weaker green colour is seen with thinner fibres containing more loosely arranged, thin fibrils. To visualise birefringence, sections were examined and captured using the Nikon EP600 microscope fitted with a polarising filter (Nikon).

Results

MTT staining of fresh skin biopsies

Optimisation of MTT staining technique on normal skin

Using initial staining conditions with incubation in 0.5mg/ml MTT solution for 1 hour, normal (unburned) skin showed positive blue-to-purple colouration for all cell types of the dermis. However, this positive staining was not uniform throughout the section, being primarily confined to the peripheries of the tissue. Overall uptake of the stain was irregular and penetration into the centre of the section was poor (Figure 1A). To improve consistency and penetration, several different staining conditions were trialled including increasing the concentration of MTT solution to 1, 2 or 2.5 mg/ml, and longer incubation times of 2, 4 or 24 hours. The staining condition with the highest concentration of MTT solution (2.5mg/ml) and the longest incubation time (24 hours) resulted in increased penetration of the stain to the centre of the section (Figure 1B). However, the improvement shown for penetration was not consistent across all normal tissue samples processed using these staining conditions. While some showed uniform staining throughout the entire section, staining of the dermis for other sections remained patchy and inconsistent (Figure 1C).

MTT staining in burned skin

Inconsistencies in stain performance were also observed for burned skin samples. For a few sections, results from staining with the MTT method were comparable to observations from H & E staining for the extent of damage to the dermis. For these sections, areas with absence of stain (non-viable cells) to the epidermis and upper portion of the dermis were clearly distinguished from stained areas (viable cells) of the deep dermis and subcutaneous tissue (Figure 2A, B). However, for the majority of sections examined, a clear line distinguishing viable from non-viable cells was not evident. Indeed, burned tissues
representative of different injury severity (e.g. superficial partial thickness and full thickness burns) were observed to have similar staining using the MTT staining method (Figure 2C, D).

![Images of different staining conditions](image1.png)

**Figure 1** Representative sections of normal (unburned) skin stained with the MTT method. **A.** 0.5mg/ml for 1 hour incubation shows some positive staining cells (blue-to-purple colouration) at the edge of the section, with less positive staining cells toward the centre of the section, indicating poor stain penetration to this region. **B.** 2.5mg/ml for 24 hours shows more uniform and intense positive staining throughout the entire section. However, this improvement in staining was not consistent across all samples processed with these staining conditions. **C.** 2.5mg/ml/24hr incubation, the upper portion of the dermis shows regular staining, whilst the deeper dermis has colourless areas with patchy and inconsistent staining. The black dashed line represents the demarcation between areas where staining is adequate from colourless areas with poor staining. Thick black arrows represent areas with increased intensity of positive staining, indicating increased metabolic activity for these cells e.g. hair follicles and basement membrane of the epidermis.
Figure 2 Variations in performance of the MTT staining method in burned skin. Results from staining with the MTT method (2.5mg/ml for 24 hours) were comparable to observations from H & E staining for the extent of dermal damage for a few burns; A. 75°C for 5 second scald showed stained areas (viable cells) in the deep dermis and subcutaneous tissue and absence of stain (non-viable cells) in the epidermis and upper half of the dermis (58% depth of dermis damaged from H & E stain); B. 80°C for 5 second scald showed absence of staining for a larger portion of the dermis (88% depth of dermis damaged from H & E stain). In contrast, for other sections a clear line of demarcation between areas of viable and non-viable cells was not evident and similar patterns for MTT staining were observed in burned skin despite differences in tissue injury severity. C. 55°C for 1 minute scald representative of a SPT burn (28% depth of dermis damaged from H & E stain) and D. 55°C for 5 minutes scald representative of a more severe FT injury (100% depth of dermis damaged from H & E stain). The black dashed line represents the depth of damage to the dermis distinguished by: lack of cell viability (colourless areas) for the MTT staining method, and depth of vascular damage to the dermis, evaluated with H & E staining.
ApopTag® staining to detect apoptosis

**Optimisation of ApopTag® staining in normal skin**

Staining using the recommended protocol for the ApopTag® kit did not produce satisfactory results in normal porcine skin tissue, compared to the positive control (inflamed human tonsil, Figure 3A). Using this protocol, a high level of background and false-positive staining of normal cells was observed (Figure 3B). The level of background staining was reduced by shortening the Proteinase K (PK) digestion time from the recommended 15 minutes. A PK digestion time of 1 minute resulted in the least amount of background staining (Figure 3C). Further to this, replacing the recommended standard stop wash buffer with a more stringent wash, SSC buffer, was shown to further reduce non-specific staining (data not shown). In normal skin, there were relatively few positively stained apoptotic cells observed.

**ApopTag® staining in burned skin**

Similar to normal skin, in burned tissue, the performance of the ApopTag® stain was highly variable. For some sections, good results were obtained using the optimised protocol. By 24 hours post-burn, for a burn representative of a SPT injury (35% depth of damage to the dermis, with H & E), compared to normal skin, an increased number of positively staining apoptotic cells were detected in the mid-dermis (Figure 4A). Generally, increased numbers of positively staining apoptotic cells were identified deep within the dermis, beneath areas showing marked tissue injury (identified with H & E sections). These apoptotic cells were observed as small clusters or single cells and were also located below or within areas showing increased cellular infiltration (Figure 4D). However, for many of the sections, false positively stained cells and artefactual staining of cellular debris remained a concern (Figure 4B, C). This decreased the value of the ApopTag® stain for identifying true apoptotic cells. For example, even within the same tissue section, variation for the pattern of staining was observed and included: discrete areas with no positive staining, other areas with clearly distinguishable positively staining apoptotic cells, and other regions with large numbers of false positive staining where most nuclei were stained.
Figure 3 Optimisation of ApopTag® staining in normal porcine skin. A. Positive control, inflamed human tonsil, stained using the recommended kit protocol. Black arrows indicate positively stained apoptotic cells. B. Normal porcine skin stained using the recommended kit protocol was observed to have a large number of normal (non-apoptotic) cells showing positive staining. C. Shortening the PK digestion time from the recommended 15 minutes to only 1 minute resulted in the least amount of background staining.
Figure 4 Variations in performance of ApopTag® staining in burned tissue. Sections at 24 hours post-burn are represented by A. 55°C for 1 minute scald and B. 55°C for 2 minute scald; and at 72 hours post-burn by C. 90°C for 5 second scald and D. 95°C for 5 second scald. True positively staining apoptotic cells stained with ApopTag® are indicated by black arrows. Open arrow heads represent non-specific false-positive staining of cells with a normal (non-apoptotic morphology) appearance. Variation between sections and within sections for the reliability of staining was observed and included: discrete areas with no positive staining, other areas with clearly distinguishable positively staining apoptotic cells, and other regions with large numbers of false positive staining where most nuclei were stained (apoptotic or not).

Sirius Red staining to detect changes in collagen morphology

*Sirius Red staining early post-burn (7 days) was not suitable for detecting change in collagen morphology*

Under polarized light, collagen fibres of normal (unburned skin) were predominantly bright red with some yellow, and the regular arrangement of thick, densely packed collagen fibres was easily recognised (Figure 5A). Early post-burn (1 hour and day 1, 3 and 7), most burned tissue showed a similar red colour birefringence to normal tissue. At these early time points, differences in the colour of collagen birefringence for normal tissue, SPT and more severe DDPT and FT burns could not be distinguished (Figure 5A-E). The only difference observed was that severely damaged burned tissue (100% depth of dermis damaged) showed less
intensity of red birefringence, with increased intermingling of dark non-birefringent fibres.

Sirius Red staining at 3 weeks post-burn allows for differences in collagen architecture between healed and non-healed burns to be evaluated

At 3 weeks post-burn, differences in the arrangement of the collagen for healed and non-healed burns were observed. By day 21, for SPT burns which had healed (≥ 95% re-epithelialised) by 21 days (75°C for 5 seconds, 60°C for 30 seconds), a red colour of collagen birefringence similar to normal unburned tissue was observed (Figure 6A, C). In contrast, for DDPT to FT burns which had not healed (< 95% re-epithelialised), 90°C for 5 seconds, 60°C for 1 minute), the upper dermis collagen fibres were observed to be thinner and more densely arranged, showing a more yellow to green birefringence. (Figure 6B, D). However, not all unhealed burns showed green birefringence of collagen. Other variables which could affect consistency of birefringence, such as, orientation of the tissue and of the polarised filter (31), were not considered. Nonetheless, the Sirius Red staining method allowed for observations regarding the organisation and heterogeneity of the collagen bundles. By day 21, in non-healed burns, collagen was less uniformly arranged and more loosely packed, with fibres appearing more crimped and thinner than those of healed burns. Similar to observations early post-burn (7 days), unhealed burns also showed increased areas of dark, non-birefringent collagen interwoven with red birefringent fibres (Figure 6D).
Figure 5 Normal skin and burned tissue at 24 hours post-burn stained with Sirius Red, viewed under polarised light to detect birefringence of dermal collagen. A. shows normal tissue. Burned tissue at 24 hours post-burn is shown for burn conditions with different % depth of dermis damaged including: B. 75°C for 5sec, 43% damage; C. 90°C for 5sec, 100% damage; D. 60°C for 30sec, 51% damage; and E. 60°C for 1min, 100% damage. Collagen in burned tissue showed a similar red coloured birefringence to normal tissue. However, severely damaged burned tissue (100% depth of dermis damaged) showed less intensity of red birefringence, with increased intermingling of dark non-birefringent fibres.
Figure 6 Sirius Red staining at 3 weeks post-burn showing differences in collagen birefringence between healed (≥ 95% re-epithelialised) and unhealed (< 95% re-epithelialised) burns. Burned tissue at 21 days post-burn is shown for burn conditions with different healing outcomes, including: A. 75°C for 5 sec, healed; B. 90°C for 5 sec, unhealed; C. 60°C for 30 sec, healed; and D. 60°C for 1 min, unhealed. For healed burns, a red colour of collagen birefringence similar to normal unburned tissue was observed. In contrast, for unhealed burns, collagen fibres in the upper dermis were observed to be thinner and more densely arranged, showing a yellow to green birefringence.

Discussion

Optimisation of the MTT staining method for freshly biopsied burned porcine tissue was not achieved in this study. Using the methodology described for staining porcine skin reported by Henze et al. (17) and Radke et al. (12), penetration of the stain throughout the section was poor. It is possible differences for stain penetration between this study and others may be explained by the size of the tissue specimens, however, biopsy size was not reported in these other studies (12, 17). Here, although the MTT staining method was not able to be
fully optimised, several approaches were found to improve penetration of MTT solution into the tissue including utilising thin tissue samples of a relatively standardised size (1–2mm thick), a longer incubation time of 24 hours, and increasing the concentration of MTT solution to 2.5mg/ml.

All cell types of the dermis were observed to stain positively (black-purple colouration) using the MTT staining method, with the pattern of staining for normal skin similar to that described by others (12, 17). Despite these encouraging results, staining in normal and unburned skin remained inconsistent. One approach suggested to improve consistency and enhance penetration of the MTT stain would be to have substantially thinner and more uniform sections for staining. An alternative method for sectioning fresh tissue for MTT staining is described by others evaluating tissue damage in mouse kidney (9), porcine myocardial tissue (13) and porcine skin (10). These studies report use of a vibrating microtome to cut the tissue into 5–10µm thick sections before incubation in MTT solution. Unfortunately, the methodological limitations and technical challenges presented by having to perform sectioning, processing and analysing of MTT stained tissue samples immediately after harvesting the tissue, were unsurmountable for this study. Instead, future avenues of research may include investigating staining methods which can be used on paraffin-embedded tissue, for example, antigen destruction immunohistochemistry (ADI). This approach has been recently described by Onul et al. (10) who suggests visualising epitope destruction using immunological staining as a comparable method to live tissue staining using the MTT method.

In this study, the TUNEL method identified increased numbers of positively stained (labelled) apoptotic cells in the dermis of burned skin at 24 hours post-burn. Similarly, using Caspase 3a stain (CC3a) in burned porcine skin, Hirth et al. (18) observed an increase in apoptotic cells at 24 hours, described as a ‘zone of apoptosis’, which approximated final depth of injury. Here, using the ApopTag® staining, a clearly defined ‘zone of apoptosis’ was not observed, however, sporadic clusters and/or single positively stained apoptotic cells were identified. These apoptotic cells were predominantly located in the dermis, below regions showing marked infiltration with inflammatory cells or evidence of severe cellular injury (determined by accompanying H & E staining). Unfortunately, due to the issues with reliability and reproducibility of staining, no quantitative analysis was performed and the overall significance of this process in burn wound progression remains unclear. While the TUNEL technique is most widely used for detection of apoptosis in tissue, it is suggested
that CC3a staining may provide earlier and improved measurement of apoptosis (32). Therefore, future investigations utilising this technique are recommended.

It is common for re-optimisation of IHC staining methods to be required between species. It is also reported that commercially available ApopTag® kits/protocols can produce high background and false-positive staining, making distinction between apoptosis and necrosis problematic (29, 33). Over digestion is known to contribute to background issues and here, decreasing the PK digestion time to 1 minute resulted in the least amount of background staining. This suggests pig skin may be more sensitive to the enzyme than mouse or human skin. The high rate of false positives and/or non-specific staining seen here has previously been well described (29, 33, 34) and is suggested to result from pre-treatment with proteinase K producing artificial strand breaks, necrosis producing strand breaks causing ‘false positive’ staining, and inconsistent fixation of tissue e.g. too extensive, incomplete, or delayed fixation contributing to non-specific staining. Additionally, this method is also subject to false positives from cells in the process of DNA repair (34), such as those partially injured or under oxidative stress. Due to the retrospective nature of this study it is unclear how inconsistencies with fixation may have affected results, however, it is probable that this hindered optimisation efforts. In future, frozen sections (which have already been collected) could be used to further investigate this process.

Staining techniques which take advantage of collagen’s natural birefringence (due to the alignment of its fibres) are described as an approach to identify changes to collagen morphology in burned tissue. Here, change in collagen birefringence, viewed with Sirius Red staining, was not a successful method with which to assess collagen damage in the early post-burn period (7 days). Burned tissue with evidence of collagen damage when observed with H & E staining (thin fibres, loss of uniformity, increased gaps between bundles) showed a similar red collagen birefringence to normal tissue. These investigations were important as they demonstrate that acutely damaged or injured collagen does not show green birefringence. Therefore, the green birefringence of collagen observed at 3 weeks post-burn for unhealed burns, is more likely to be associated with new re-growing or re-modelling collagen fibres rather than damaged fibres. This suggests that severe burns taking longer than 3 weeks to fully re-epithelialise, have increased disruption to their collagen architecture necessitating greater re-modelling compared to burns which had fully re-epithelialised (healed). Whether this increase in re-modelling of collagen in unhealed burns is associated with an increased likelihood of hypertrophic scar formation is not investigated here.
Nonetheless, results presented here illustrate the usefulness of Sirius Red staining for evaluating the organisation and heterogeneity of collagen fibres in burn wounds as early as 3 weeks post-burn. Future studies, using IHC staining or multi-photon microscopy to detect collagen subtypes I and III could be considered as an approach to further examine differences between these healed and unhealed burns.

There are several markers of injury identified in the literature as potentially useful for the analysis of burn wounds. In future, investigation of other markers of injury may include:

- HMGB1, to detect necrotic cells
- CC3a, to detect apoptotic cells
- IHC staining for collagen subtypes I and III

**Conclusion**

This study examined different histopathological staining approaches for their reliability in identifying pathological changes in cells and tissue damage with respect to previously characterised histological damage and wound healing of different burn conditions. Overall, the staining techniques used proved to be unreliable and inconsistent. Results from this study serve to highlight the general utility of standard H & E staining, which is the mainstay of burn histopathological analysis. However, this study was limited to investigating only a few out of the large number of potential histochemical and immunohistochemical stains described in burn literature.

**References**


