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ANTHONY PAPP

Experimental Thermal Injury

New Methods in Assessing Tissue Damage

Doctoral dissertation

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ABSTRACT

A series of studies was carried out in order to establish an experimental burn model to create superficial, partial thickness and full thickness cutaneous burns in pigs as well as to evaluate the applicability of the microdialysis method and the dielectric measurements in burn wound research.

Contact burns to pigs were created with a brass plate in all studies under general anaesthesia. In the methodological study, the histological examination of tissue biopsies showed that the 1 second contact resulted in a superficial thermal injury limited to the superficial third of the dermis, the 3 second contact in a partial thickness injury extending to the middle third of the dermis and 9 seconds to a full thickness injury reaching the subcutaneous fat. The repeatability of burn depth creation yielded a kappa coefficient 0.92. These contact times were chosen for the further studies.

The tissue concentrations of histamine, one of the most important local mediators after thermal injury, were determined by using the microdialysis method which enables continuous collection of samples from the extracellular fluid for analysis. Our study showed a time-dependent correlation of tissue histamine concentrations at 1 and 2 hours post injury. The full thickness injury resulted in higher initial histamine concentrations in tissue than the partial thickness and superficial burns. Histamine concentrations decreased in all burn sites until 6-12 hours, but there was a late secondary increase in the tissue concentrations at 24 hours without an increase in the histamine concentrations in plasma. Hence, the microdialysis method proved to detect changes in histamine concentrations in tissue and proved to be an applicable method in collecting samples from burned tissue.

When performed with 300 MHz, the dielectric measurements specifically give information about the amount of both bound and free water (= total water) in tissue. The measurements are non-invasive, painless and instantaneous. The performed measurements in our study showed that the partial and full thickness burns could be differentiated during the first 24 hours post injury with this method according to their edema forming characteristics. This is of great clinical interest, because clinically burn depth determination is done at 48-72 hours post injury. Also, a marked edema was found in the subcutaneous fat in all burns. The findings of increased water amount in fat were confirmed in the last study with lyophilization.

These studies showed that a reproducible experimental burn model was obtained and that the microdialysis method and the dielectric measurements are applicable in burn wound research. The microdialysis method has a great potential in examining accumulation of local mediators in burn wounds and the dielectric measurements is of clinical interest in early burn depth determination.

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Medical Subject Headings: animal experimentation; burns; edema; dielectric impedance; erythrocytes; histamine; histology; microdialysis

To Ulla, Béla, Beata, Bettina and Benjam

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Abbreviations

1S	1 second burn site
3S	3 second burn site
9S	9 second burn site
AUC	area under the curve
C1	complement 1
cmH_2O	centimetres of water
FiO_2	fraction of inspiratory oxygen
i.v.	intravenously
IgE	immunoglobulin E
kDa	kilodalton
MBq	megabecquerel
MHz	megahertz
nmol/l	nanomoles per litre
NPV	negative predictive value
$paCO_2$	partial pressure of carbon dioxide
PPV	positive predictive value
R	correlation coefficient
RBC	red blood cell
ROC	receiver operating characteristic
rpm	rounds per minute
SD	standard deviation
SE	standard error

LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following articles referred to in the text by their Roman numerals:

I Papp A, Kiraly K, Härmä M, Lahtinen T, Uusaro A, Alhava E: The progression of burn depth in experimental burns: a histological and methodological study. Burns 2004; 30(7): 683-9.

II Papp A, Härmä M, Harvima R, Lahtinen T, Uusaro A, Alhava E: Microdialysis for detection of dynamic changes in tissue histamine levels in experimental thermal injury. Burns, accepted.

III Papp A, Lahtinen T, Härmä M, Nuutinen J, Uusaro A, Alhava E: Dielectric measurement in experimental burns: A new tool for burn depth determination? Plastic and Reconstructive Surgery, accepted.

IV Papp A, Romppanen E, Lahtinen T, Uusaro A, Härmä M, Alhava E: Red blood cell and tissue water content in experimental thermal injury. Burns, submitted.

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This thesis also contains unpublished data.

CONTENTS

1. INTRODUCTION

It is estimated that the incidence of burn trauma leading to hospitalization is 23.5/10000/year in Finland. Within the 1200 patients who require hospitalization annually, 590 require operative treatment and 50 need intensive care due to thermal injury (Papp et al 2001). Even though more than 1 million people are burned in the United States each year and 5500 die as a result of their burn injury (Brigham and McLoughlin 1996), there is still no agreement on how to perform the final burn depth determination dictating burn treatment.

Several different animals have been used in experimental burn research (Converse et al 1965, Leape 1968, Zawacki 1974a, DeCamara et al 1982, Chvapil et al 1984, Kaufman et al 1990, Brans et al 1994, Nanney et al 1996, Cribbs et al 1998, Knabl et al 1999a, Singer et al 2000). Most of these have been mice, rats, guinea pigs or rabbits, whose skin differ greatly from human skin. With several similarities in the anatomical structure and skin thickness to humans, porcine skin is ideal for burn wound research (Douglas 1972, Meyer et al 1978, Brans et al 1994, Singer et al 2000, Paddock et al 2003). It is also an animal large enough to create several uniform burns. Previously, experimental burns have been inflicted to the thick dorsal paraspinal skin of the pig (Brans et al 1994, Nanney et al 1996, Paddock et al 2003) even though the thickness of the ventral skin in pigs is closer to that of human.

Thermal injury induces several alterations in tissue. Progression of burn depth, edema formation, changes in blood flow and the liberation of different mediators play an integral role in the final outcome of burn wound healing. From the several compounds found in tissue after burn trauma histamine is one of the most important local mediators. Previously, the histamine concentration in tissue has been determined by analyzing tissue biopsies (Suzuki et al 1971, Fazekas et al 1973, Horakova and Beaven 1974, Boykin et al 1980). Recently, a continuous collection technique of samples from the extracellular space has been obtained with the microdialysis method. Determination of histamine concentration in tissue with this technique has been used both in humans (Anderson et al 1992) and in animals (Groth 1996). It has not, however, been reported in burn wound research.

Local edema formation is characteristic for burn injuries. Several different techniques in studying burn-related edema have been reported (Leape 1968, Heydinger et al 1971, Björk and Arturson 1983, Demling et al 1984, Löfgren et al 1998, Lindahl et al 1993, Zdolsek et al 1998). However, these techniques are unable to quantitatively assess the amount of edema in different layers of tissue. The dielectric measurements are able to provide this data. The measured value, the dielectric constant, is directly related to the water amount in tissue and can be measured from different depths of tissue with probes of different sizes (Alanen et al 1998, Nuutinen et al 1998).

It is known that burns of different depths result in edema formation in different magnitudes, but the burn-related edema formation in different depths of skin has not been described.

Burn-related animal research has not been done in Finland since the 1970's. (Asko-Seljavaara 1974, 1975). The present study series was carried out firstly to create a reproducible animal model for obtaining burns of different depths and secondly to determine whether the microdialysis method and the dielectric measurements are applicable in burn wound experiments. Special attention was paid to the ability to differentiate burns of different depths with these methods.

2. REVIEW OF THE LITERATURE

2.1. Skin anatomy

Skin is the largest organ in humans weighing ca 4 kg in adults. It is responsible for thermoregulation and sensation, acts as a barrier against external factors and is an important component of the body's immune system (Figure 1).

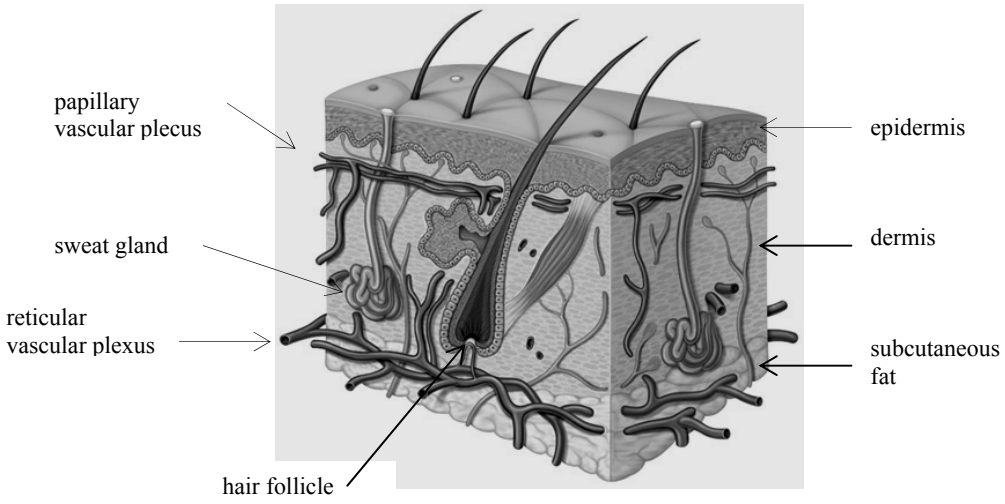


Figure 1. Skin anatomy. Modified with the permission of Mr Richard Tibbits, AntBits Illustration (www.antbits.co.uk).

2.1.1. Epidermis

The total thickness of human epidermis varies from 50 to 120 μm (Meyer et al 1978). In the five layers of epidermis cells undergo division and differentiation during their lifetime. Cell division takes place in the deepest part of the epidermis called the basal cell layer, while the differentiation occurs in the overlaying layers. The stratum spinosum is the layer above the basal cell layer consisting of keratinocytes responsible for the production of fibrous keratin protein, e.g., keratinization. Superficially to this is the stratum granulosum, a 4-5 cell layered structure where cells gradually loose their form, become flattened and finally the nucleus and intracytoplasmic organelles degenerate. In the next layer, stratum lucidum, the cell is prepared to move into its final sublayer with the addition of melanin granules. This layer is well seen only in thick skin. The stratum corneum is constructed of dead cells and it forms the outermost layer of skin (Kessel 1998a). This part represents about 25 % of the total thickness of the epidermis with 15-100 layers of large, flat cells filled with keratin. This provides a semi-permeable physical

barrier against the outer environment. Epidermis also consists of melanocytes in the basal cell layer producing pigment and protecting skin from the ultraviolet radiation, Langerhans cells as a part of the immunologic defence function of skin and Merkel cells which are believed to be involved with the sensation of fine touch.

2.1.2. Dermis

The interface between the epidermis and dermis is called the basement membrane. Dermal thickness varies from <1 mm in the eyelids to >5 mm in the posterior trunk (Heimbach et al 1992). It is mainly composed of collagen and elastic fibres, glycosaminoglycan and tissue water. The cells in the dermis are mostly fibroblasts but also macrophages, mast cells, platelets and endothelial cells are found. Collagen and elastin represent approximately 75 % and 4 % of the dry weight of the dermis, respectively.

In the superficial papillary dermis the collagen fibres are fine-structured and loosely packed, while in the deeper reticular dermis they are thick and densely arranged (Pasyk et al 1989). Papillary dermis contains anchoring rete pegs against the epidermal rete ridges and is the most biologically active part of the dermis. The reticular dermis is responsible for durability and anchoring of the skin appendages. Elastic fibres are mainly located in the reticular dermis.

2.1.3. Skin vasculature

Blood circulation through skin serves two functions: nutrition of the skin and regulation of the body temperature by conduction of heat from the internal structures of the body to the skin, where it is lost by exchange with the external environment. The cutaneous circulation comprises two types of vessels: the nutritive vessels and the vascular structures concerned with heat regulation. The latter includes an extensive subcutaneous venous plexus which can hold large quantities of blood and arteriovenous anastomoses. At ordinary skin temperature the amount of blood flowing through the skin is about 10 times higher than is required for the nutrition of the skin.

There are two main vascular plexuses in the dermis. The superficial papillary dermis is highly vascularized with 60-75 capillary loops/mm^2 (Hopewell 1990). The capillary density is 12-14 times that of the deeply situated reticular dermis. The diameter of the blood vessels in the papillary region is typically 10-20 μm and about 50 μm in the reticular dermis. In the papillary dermis the endothelial cells of the arterioles are covered typically with one or two layers of smooth muscle when there are 4-5 layers in the reticular dermis.

2.1.4. Skin appendages and subcutaneous fat

In humans, the skin appendages include hair, nails and several types of secretatory glands. Together, the skin and the skin appendages are known as the integumentary system of the body. An adult human has 1.6 – 4.0 million sweat glands. Most of them are eccrine sweat glands which are found all over the surface of the body being most numerous on the palms and soles. They originate deep in the dermis and connect to the surface of the skin by a coiled duct. The cells in the base of the gland secrete sweat. The apocrine sweat glands are less numerous and are located around the areolae, genital area and armpits. They are also situated deep in the dermis, but open into hair follicles. Their function remains unclear. Sebaceous glands are most numerous in the face, scalp and upper trunk and are absent from the palms and soles. Most of them open into hair follicles, also. The secreted substance called sebum helps to keep the hair and skin from drying. The subcutaneous layer lies between the dermis and the underlying muscle fascia covering muscle. It contains groups of adipocytes which are separated by fibrous septa. It serves three functions: to insulate the body from cold, to absorb trauma and to act as storage for the body's reserve fuel.

2.2. Pig skin as a model for human skin

The skin of domestic mammals is becoming increasingly important in biomedical research as a model for experimental research on human skin (Meyer et al 1978). Several different animals have been used for burn research (Converse et al 1965, Zawacki 1974a, DeCamara et al 1982, Chvapil et al 1984, Kaufman et al 1990, Brans et al 1994, Nanney L 1996, Cribbs et al 1998, Knabl et al 1999a and 1999b, Singer et al 2000). Pigs were used in the present study since pig skin has many similarities to human skin (Douglas 1972, Meyer et al 1978, Brans et al 1994, Singer et al 2000, Paddock 2003) and its skin best fulfils the requirements of a model for human skin (Meyer et al 1978). Firstly, the absence of the intradermal muscular layer, panniculus carnosus, in pigs leads, as in humans, to closure of the wound by epithelial growth instead of contraction as in rodents. The interaction between the panniculus carnosus and the overlying dermal and epidermal layers is not fully understood and, therefore, may confound extrapolation of data obtained from rodents to humans (Paddock 2003). Secondly, pig and human skin have the same relative thickness of epidermis (70-140 μm and 50-120 μm, respectively) and dermis, a presence of epidermal ridges, a distinct papillary dermis, similarities in both the vascularization of the hair follicle and the structure of the collagenous tissue framework and a deep layer of subcutaneous fat (Meyer et al 1978). The vascular density in the deep dermis is similar in humans and pigs (Meyer et al 1978, Young and Hopewell 1980) but is less dense in the papillary plexus

in pigs (Meyer et al 1978). Pigs, like humans, have 'fixed skin', that adheres tightly to subdermal structures and have a similar deposition of subdermal fat (Paddock et al 2003). Dorsal skin hair in pigs undergoes sporadic hair growth and replacement similar to humans. On the other hand, porcine skin does not contain apocrine sweat glands, which in humans contain cells that participate in regeneration of the epidermis following partial-thickness injuries. However, it does not appear to appreciably alter healing of pig wounds compared to human wounds (Paddock et al 2003).

In experimental burns, it is important to choose an animal big enough to have flat surfaces to create uniform burns (Singer et al 2000) and have control sites in the same animal (Paddock et al 2003) thus reducing the interanimal variation. Previously the thick dorsal paraspinal skin of pig has been used in burn wound research (Brans et al 1994, Nanney et al 1996, Paddock et al 2003). The thinner ventral body skin has been used previously in small piglets (Chvapil et al 1984). The thickness of the ventral, rather than the dorsal, non-burned body skin in pigs resembles that of human. Previously, the skin thickness in 3-4 month old pigs has been measured to be ca 1.4 mm (Hopewell et al 1989). This is close to the thickness of human skin (1.44 $\pm$ 0.25 mm) measured from the thigh and forearm of 25-66 year old people (Raju et al 2003).

2.3. Mast cells

2.3.1 General

Mast cells and basophils were first described by von Reclinghausen and characterized by Paul Erlich more than 100 years ago as granular cells that stain metachromatically with basic dyes. Mast cells are found in connective tissues whereas basophils are present in blood. Mast cells are derived from multipotential stem cells that are in the bone marrow. The precursors migrate into blood and other tissues and appear as ungranulated lymphoid cells. The mature cells do not circulate, are long-lived and retain the capacity to proliferate (Siraganian 1994). Mast cells are widely distributed in the connective tissue of skin and in mucous membranes of the digestive and respiratory tract, especially near small blood vessels (Kessel 1998b). In normal skin, mast cells occur in the greatest density in the superficial dermis (Church and Clough 1999). Both mast cells and basophils synthesize and store histamine, proteoglycans and proteases within their granules and have surface receptors that bind IgE with a high affinity. The binding of antigens to this IgE activates the cells to degranulate various mediators. Human mast cells contain 5-20 μg of histamine per 10^6 cells (Siraganian 1994). Thermal trauma causes degranulation of mast cells and histamine liberation (Kessel 1998b, Friedl et al 1989). Mast cell granules in pigs are distinct from that in humans. In pigs granules are of uniform size and contain amorphous granular material of

variable electron density whereas in humans structures are recognized within granules permitting definition of subtypes of mast cells (Xu et al 1993). Porcine mast cells either don't contain chymase or pig chymase does not react with the substrate normally used for human chymase (Xu et al 1993).

The release of mediators from mast cells after stimuli is a rapid secretory process that is complete in less than 30 minutes (Siraganian 1994). The membrane of the secretory granule fuses with the plasma membrane and is associated with swelling of the individual granules (Schwartz and Austen 1982). Fusion of granules with each other and with the plasma membrane forms channels. The secretion of mediators requires energy which is acquired from the membrane potential changes following cell stimulation (Siraganian 1994).

2.3.2. Histamine

Histamine (β-imidazolyl-ethylamine, molecular weight 111 Da) is formed by mast cells in the Golgi apparatus from the amino acid L-histidine by histidine decarboxylase (Schayer 1963). It is stored in the secretory granules of mast cells in combination with the acidic glycosaminoglycans of heparin. Solubilization of histamine with degranulation of mast cells occurs by cation exchange with extracellular sodium. Histamine exerts its effects on cells and tissues by binding and activation of cell surface receptors which have been designated H1 and H2 (Ash and Schild 1966).

Histamine is widely distributed throughout the various tissues of the human body. Most of the histamine is stored in the secretory granules of mast cells; a finding discovered 1952 by Riley and West (1952). In blood, histamine is contained in the basophils, being the most mobile source of histamine. It is released by the immunological activation of these cells in immediate hypersensitive reactions. After histamine is released it binds and activates specific receptors on other cells that result in the contraction of bronchial and gastrointestinal smooth muscle and enhanced vascular permeability (Siraganian 1994). Histaminergic neurons of the central and peripheral nervous systems constitute an important non-mast cell source of histamine (Pearce 1992). In addition to mast cells, histamine in human is found in basophils and histaminergic nerves and also in the gastric enterochromaffin-like cells (MacGlashan 2003).

Histamine secretion from the mast cell may be induced by a large number of immunological and pharmacological stimuli. The synthetic polyamine compound 48/80 (Horner and Winkelman 1968) and the neuropeptide substance P (Lowman et al 1988) are probably the most widely used chemical histamine liberators in research. Thermal injury has shown to cause rupture of the secretory granules of mast cells causing liberation of histamine (Friedl et al 1989) resulting in a rise in tissue histamine concentrations (Suzuki et al 1971, Fazekas et al 1973). Histamine

concentration in plasma increase within 1 minute after thermal injury and the increase is proportional to extent of the surface area injured (Yurt and Pruitt 1986). Full thickness burn has more extensive immediate loss of blood supply, which leads to less immediate but more sustained release of histamine. Plasma concentration of histamine immediately after burn appears to be directly related to the extent of the surface area injured and the time-related profile of plasma histamine concentration is related to the depth of the injury (Yurt and Pruitt 1986). The early rise in blood histamine in cutaneous burns is primarily due to the release of histamine from the skin (Suzuki et al 1971). Thermal injury causes complement activation with anaphylatoxin release and histamine secretion from mast cells, leading to enhancement of xanthine oxidase activity and increased production of oxygen radicals which damage endothelial cells resulting in increased vascular permeability (Friedl et al 1989). Histamine is most likely the predominant vasoactive substance responsible for the increased permeability seen after thermal injury (Boykin et al 1980). Venular endothelial cells contract causing large endothelial gaps. Histamine can also cause a rise in capillary pressure by arteriolar dilation and venular contraction (Markley et al 1975).

Tissue histamine concentrations have previously been measured from excised skin samples (Sanyal 1962, Suzuki et al 1971, Horakova and Beaven 1974). This makes it impossible to determine repetitive analyses from the same site. The microdialysis technique, however, enables continuous collection of samples from the extracellular space. Hence, this novel technique might provide additional information of the local secretion of histamine, or other mediators, at the burn site.

2.4. Thermal injury

2.4.1. Local effects of heat in tissue

Thermal energy or heat is a manifestation of random molecular kinetic energy. This energy is transferred from high-energy molecules to those of lower energy during contact via a process referred to as conduction. When contemplating the effect of heat on a cell, one must consider the temperature to which the cell is exposed as well as the time period for which the temperature is sustained. These two factors together determine the degree of cell damage. Moritz and Henriquez (1947) have presented the time-temperature relationship in thermal injury to skin.

Early denaturation of proteins occurs at temperatures between 40 °C and 44 °C. Cellular functions are impaired leading to a high intracellular Na+ concentration and concomitant swelling. At temperatures higher than 44 °C, if contact time is long enough, an eventual cell necrosis results. As a part of the damage process oxygen free radicals are produced promoting

further cell membrane damage. If the cell is rapidly cooled, a significant amount of the damage is eluded (Raine et al 1981).

Ensuing protein damage leads to coagulation of proteins and furthermore to a complete cell necrosis. In skin this process begins from the surface where the temperature is the highest, extending then deeper into the tissue. The area of skin which is burned to complete necrosis is called the zone of coagulation and is the first of the three burn zones described by Jackson (1953). This central zone is characterized by a complete obliteration of vessels in the subpapillary plexus and represents irreversible necrotic injury. Deep and peripheral to the zone of coagulation is the zone of stasis, where the cells are initially viable. Here, however, progressive impairment of vascular flow leads to tissue ischemia which might be devastating for already compromized cells. Impaired blood flow is due to platelet microthrombous formation, neutrophil adherence to vessel walls, fibrin deposition, endothelial swelling and vasoconstriction (Boykin et al 1979). Impairment of blood flow ensues within a couple of hours in more severely burned areas and is delayed for up to 16-24 hours in less severe regions (Zawacki 1974a). Early in the recovery period, the zone of stasis can convert to either a zone of coagulation or preferably to a zone of hyperaemia. The peripheral zone of hyperaemia is characterized by capillary vasodilatation due to vasoactive mediators produced in the course of inflammatory response showing no apparent structural damage to the dermis.

2.4.2. Edema formation

Burn wound pathology is characterized by an inflammatory reaction leading to a rapid edema formation due to dilatation of resistance vessels with increased effective transcapillary filtration pressure, increased extravascular osmotic activity in damaged tissue and increased microvascular permeability to macromolecules (Arturson et Jonsson 1979). Increased permeability of blood vessels after thermal injury was established by Cohnheim as early as 1873. The first direct proof of increased microvascular permeability for plasma proteins after thermal injury, however, was given by Netsky and Leiter (1943), who demonstrated the passage of horse serum across the capillary endothelium of the dog after a subaxillary full thickness scald burn. An altered rate of passage of protein in the unburned area was also noted. These findings were confirmed by Cope and Moore (1944) using radioactive dyes after a minor scald injury in dogs. An immediate and delayed capillary response to burn injury was described by Sevitt (1958), where the immediate reaction was of short duration and was considered to be a direct effect of heat lasting for 1-2 minutes. The delayed response, however, was found in dermal capillaries in minor burns and in the subcutaneous fat in more severe burns at 1-2 hours post injury. The delayed capillary permeability disorder was thought to be an indirect effect mediated by chemical or neurochemical

means. The immediate reaction was inhibited by antihistamines but the delayed response was not. Hence, the part played by the release of histamine in the response of skin vessels to heat was considered not important in the delayed capillary permeation. The immediate increase in permeability is thought to be due to the endothelial cell gap formation instead of endothelial damage itself in superficial burns and at the edges of deeper burns (Cotran and Remensnyder 1968). These openings have been found to relate to the contraction of the endothelial cells (Joris 1972). On the other hand, permeability disorder in full thickness burns is related to direct damage of vessel walls caused by thermal injury (Cotran and Remensnyder 1968).

Several studies have been performed in order to identify the location of the permeability disorder within the vascular tree. Immediate damage has been demonstrated in the venules (Wells and Miles 1963, Spector et al 1965, Cotran and Remensnyder 1968, Vegad and Lancaster 1973) and the delayed permeability disorder either in the capillaries (Wells and Miles 1963, Spector et al 1965) or both in the capillaries and venules (Ham and Hurley 1968, Cotran and Remensnyder 1968).

Under normal physiological conditions, the pressure of arterioles, capillaries and venules causes a filtration of fluid into the interstitial space of all tissues. This fluid is then partially reabsorbed into the circulation, while the remaining net filtration is removed by lymphatic drainage. Edema develops when the rate by which fluid is filtered out of the vessels exceeds the flow in the lymph vessels draining the same tissue mass (Demling et al 1978).

In addition to the reported changes in permeability, other components of edema formation have also been suggested. It has been proposed, that edema formation is rather a pressure-driven entity with recordable changes in capillary and interstitial pressures. Negative interstitial fluid pressure after thermal injury has been verified in both experimental and human burns (Lund et al 1989). This acts as a suction force called the imbibition pressure. Collagen degradation and water solubility increased with extension of the heat exposure time. Thermal degradation of collagen seemed to be the main mechanism responsible for the generation of increased imbibition pressure.

Fluid transportation is quantitatively described by the Landis-Starling equation:

$$\mathbf{J_v = K_f\,((P_c - P_{if}) - \sigma(\pi_p - \pi_{if}))}$$

J_v = the volume of fluid that crosses the microvasculature barrier
K_f = capillary filtration coefficient
P_c = capillary hydrostatic pressure
P_{if} = interstitial fluid hydrostatic pressure
σ = osmotic reflection coefficient
π_π = colloid osmotic pressure of plasma
$\pi_{\phi\iota}$ = colloid osmotic pressure of interstitial fluid

The colloid osmotic pressure of plasma is normally 25-30 mmHg and diminishes during extravasation. The interstitial colloid osmotic pressure is about half of the colloid osmotic pressure of plasma. Edema appears when oncotic pressure in plasma is lower than 2-2.5 kPa (15-19 mmHg) (Zetterström and Arturson 1980).

2.4.2.1. Measurement of edema

Several methods of measuring tissue edema have been described. The easiest way is the fluid displacement method (Björk and Arturson 1983) using either water or mercury. The amount of water in tissue can be measured by excising the burned tissue and drying it with different methods. Calculating the difference between the wet and dry weights gives the amount of water in tissue (Leape 1968). This technique, however, requires removal of the tissue from the original site. The thermogravimetric analysis (Heydinger et al 1971) provides continuous recording of the weight of the sample as a function of the temperature. The dichromatic absorptiometry (Demling et al 1978) non-invasively determines the masses of tissue components by photon transmission measurements. The water and protein components are essentially indistinguishable by this technique and therefore it does not give an accurate estimation of the amount of water. The changes in the volume of tissue can be measured with a plethysmograph (Löfgren et al 1997). The so-called impression method evaluates tissue edema by measuring the resistive force of the tissue under compression (Lindahl et al 1993). The bioelectrical impedance measures the resistance and the reactance of a tissue conductor to application of an alternating electric current (Zdolsek et al 1998). This method gives a general picture of the water amount in total body but is unable to detect local changes. Also ultrasound (Kalus et al 1979) and nuclear magnetic resonance (Koruda et al 1986) have been used to evaluate burn-related edema formation but have not reached clinical significance.

None of the above mentioned methods can provide us with a numeric value of tissue water repetitively from the same location and from a certain depth of tissue. The dielectric measurements, however, provide this information. The measured value, the dielectric constant, at high radiofrequency is directly related to total tissue water (Foster and Schwan 1989). As the water amount can be estimated non-invasively from different layers of skin and subcutaneous fat, it is likely that the dielectric measurements add our knowledge of edema formation in tissue after thermal injury.

2.4.3. Changes in local blood flow

Restoration of local blood flow after thermal injury is crucial for burn wound healing. Several blood vessel –related changes have been reported subsequent to burn injury. Agglutination of red blood cells and platelets (Arturson and Wallenius 1964), leucocyte sticking to vessel walls (Olofsson et al 1973, Mulligan et al 1994) and the liberation of vasoactive substances (Arturson and Jonsson 1979) have been reported. Massiha and Monafo (1974) pointed out the importance of venous blood flow at burned sites. They thought that progressive changes in the venous side of the dermal circulation leads ultimately to thrombosis and that interstitial extravasation may be of primary importance in the pathogenesis of dermal ischemia following thermal injury. Owen and Farrington (1976) found a temperature-dependent increase in blood flow within 1 minute post burn it rat paw by using radio labelled ^{51}Cr red blood cells. After the primary increase, the blood flow decreased to a level of 4-5 times greater than control sites in 15 minutes. Löfgren et al (1997) demonstrated a biphasic increase in circulation in burned rat skin with laser Doppler velocimeter. The first rise was up to 400 % during the first 5 minutes post burn which stabilized to a level of 100 % above pre burn levels at 60 minutes. Increased blood circulation measured by using radio labelled red cells has been found also in brain, heart, the hepatic artery and the adrenal glands in very large, 70 %, full thickness burns in guinea pigs (Ferguson et al 1977). However, no changes were found in the diaphragm, liver, spleen, gastrointestinal tract, muscle or bone. The pancreas, subcutaneous fat and skin, both burned and non-burned, had decreased blood flow. These findings in fat have also been demonstrated in dogs (Hamar et al 1979) but also a biphasic reaction of fat blood flow has been described by cytometry in sheep (Sakurai et al 2002) where the initially decreased blood flow was followed by a late hyperaemic state at 72 hours post injury.

The site of vascular injury has been widely examined. Nanney (1982) demonstrated with electron microscopy that large intercellular gaps were present 15 min after immersion injury in the venular endothelium in guinea pigs and at 30 min in the capillary endothelium, too. These changes were detectable throughout the 24-hour-study. Aggraval et al. (1990) found in rat studies that arteriolar and venular dilations were directly and inversely proportional to the size of the vessel, respectively. Smaller arterioles dilated only 13 % and the bigger ones 58 %, smaller venules dilated 56 % and the bigger ones 12 %.

The beneficial vascular effects of cooling (Raine et al 1981, Kaufman et al 1990) and several drugs have been reported. Burn wounds cooled by 30 minutes presented markedly better healing than control animals and animals cooled 60 min after burning (Raine et al 1981). Heparin was beneficial only when given pre burn (Noble et al 1977) but ibuprofen and imidazole (Ehrlich 1984) inhibited microvascular occlusion given as late as 6 hours post injury by inhibiting

prostacyclin deliberation to dermal vasculature. The beneficial effects on dermal ischaemia of topical methylprednisolone acetate (Robson et al 1978), prostaglandin synthesis inhibitors indomethacin and acetylsalicylic acid (Del Baccaro et al 1978), thromboxane inhibitors (Del Baccaro et al 1980), ibuprofen (Regas and Ehrlich 1992, Barrow et al 2000), hyperbaric oxygen (Germonpré et al 1996), C1-inhibitor (Henze et al 1997), lidocain and prilocain (Jönsson et al 1998) and subathmospheric pressure (Morykwas et al 1999, Kamolz et al 2004) have been documented. Negative effects of different treatment modalities have also been reported. Blomgren and Bagge (1984) found that cooling or pre burn medication with cimetidine, ketanserin, methylprednisolon or indomethacin did not have any influence in the formation of post burn necroses in mouse ears. Infection, drying (Zawacki 1974a), hypernatremia (Kuroda et al 1997) and systemic application of a vasoconstrictor (Knabl et al 1999a) have been found to increase burn depth.

2.4.4. Burn depth determination

2.4.4.1. Clinical observation

Despite modern technology, clinical observation still remains the standard for burn depth estimation (Heimbach et al 1992). The most superficial (first and superficial second degree) and the deepest full thickness burns are not a challenge for clinical burn depth determination. Superficial dermal burns form blisters with fluid accumulation under the epidermis. Blister formation may be late in these burns and occur only during the second post burn day. Once the blister is removed, the underlying wound is red, wet and painful and blanches under pressure (Zawacki 1974b). Partial thickness and deep dermal burns blister also, but the blistering is more immediate. The underlying wound is usually a mottled pink and white in colour, but eventually turns whiter in time in deep dermal burns. In the early phase blanching under pressure may be noticed with slow or no refill of capillaries. These burns are not as painful as the superficial dermal burns due to the partial destruction of nerve endings. The full thickness burns are leathery, firm and depressed when compared to adjoining normal skin. They have a dry surface, can be presented with several different colourings and may have translucent clotted vessels visible.

In addition to clinical observation, the other techniques used in burn depth determination take advantage of the ability to detect (1) dead cells or denaturated collagen (by biopsy, vital dyes), (2) changes in blood flow (by fluorescein, laser Doppler, thermography), (3) the colour of the wound (by light reflectance) or (4) other physical changes, like edema (by nuclear magnetic resonance, ultrasound).

2.4.4.2. Histology

Histology has been considered the gold standard for burn depth determination. Several different staining methods have been described. The most used in burn research is the haematoxylin-eosin staining (Kahn et al 1979, Chvapil et al 1984, Brans et al 1994, Cribbs et al 1998, Knabl et al 1999b, Watts et al 2001, Singer et al 2000), but also the van Gieson (Brans et al 1994), the Massons trichrome (Chvapil et al 1984, Brans et al 1994, Watts et al 2001) and the vimentin immunostaining (Nanney et al 1996) have been used. The use of histology in clinical settings, however, is controversial. Biopsies leave permanent scars, are time-consuming and expensive and require an experienced histopathologist to distinguish live from denaturated collagen and cells. The biopsy site needs to be anaesthetized leading to swelling to the area. Also, the burn wound is a dynamic entity and the damage ensues at least 48 hours (Hinshaw 1968, Singer et al 2000) being the maximum at 8 hours post injury (DeCamara et al 1982). Therefore, the timing of the biopsy is troublesome. In addition, there is no guarantee that the areas adjacent to the biopsy are of the same depth.

There are several histological findings in the skin after thermal injury: separation of the epidermis from the basal cell layer (epidermal blistering), distortion of cell contour in the epidermis, dermis, appendages and vessel walls. The following criteria has been used for blocked vessels: (1) vessels tightly packed with erythrocytes that have clearly undergone cellular degeneration, (2) loss of endothelial cell lining associated with debris within the lumen and (3) destruction of vessel walls with or without surrounding extravasation of the erythrocytes (Watts et al 2001). Patent vessels have intact vessel walls with normal endothelial cells with no debris or tightly packed erythrocytes within the lumen.

2.4.4.3. Other methods

India ink has been used in experimental burn research to demonstrate blood flow to the skin. A volume 2.5 times the total blood volume of the animal is injected into the aorta at a constant pressure of 400 mmHg (Zawacki 1974a, Kaufman et al 1990) to fill the dermal vascular plexuses but it is not of clinical interest as it kills on injection. Methylene blue (Davies et al 1989) together with silver sulphadiazine has been tested in clinical trials. It causes a discoloration of dead tissue and was thought to aid excision. However, it has never become useful in practise. Systemically injected fluorescein is delivered via patent blood vessels and fluoresces under an ultraviolet lamp. It has not reached clinical significance due to its incapability to distinguish between partial thickness and deep burns (Black et al 1986). Laser Doppler flowmetry has been used in several experimental and clinical trials to monitor cutaneous circulation and has reached a wide-spread use clinically. The confounding factors, however, are several including changes in body

temperature, state of anxiety and elevation of an extremity. On the other hand, serial measurements and the scanning laser Doppler have improved the accuracy of the measurements (Mileski et al 2003) making laser Doppler the most widely used mechanical tool in burn depth evaluation at the moment. Some reports have been published also on thermography (Cole et al 1990), ultrasound (Kalus et al 1979), nuclear magnetic resonance (Koruda et al 1986), surface temperature (Wyllie and Sutherland 1991) and light reflectance (Svaasand et al 1999). Ultrasound detects the interface between thermally denaturated and normal collagen (Cantrell and Yost 1984). The use of ultrasound to determine burn depth by detecting denaturated collagen is controversial, because collagen denatures at 65ºC while the epidermal cells, from which the burn must heal, are killed already at 44-47ºC, so the ultrasonic burn depth evaluation is likely to be underestimated (Heimbach et al 1992). The non-contact ultrasonography, however, has given some promising results in predicting burns that heal within 3 weeks (Iraniha et al 2000).

3. AIMS OF THE STUDY

The purpose of this study was to investigate local tissue events after experimental thermal injury in pigs with special interest in the microdialysis technique and the dielectric measurements.

The specific aims were:

1) To create a reproducible model for obtaining superficial, partial and full thickness cutaneous burns in pigs.

2) To evaluate the feasibility of the microdialysis method in experimental burns to collect samples for histamine analysis.

3) To study whether the dielectric measurements will provide a sensitive method for examining tissue edema after experimental thermal injury at different depths of tissue.

4) To examine whether the dielectric measurements enable early differentiation of partial and full thickness burns in pigs.

5) To explore the correlation between the dielectric constants and absolute tissue water contents after thermal injury.

4. MATERIALS AND METHODS

These studies were approved by the Institutional Animal Care and Use Committee of the University of Kuopio. The thermal injuries were created to 11 animals in two successive study periods. Original studies I and III consisted of 6 animals and studies II and IV of 5 animals (Table 1).

	Publication					
Animals	**I**		**II**	**III**	**IV**	**Follow-up (h)**
	Study 1	*Study 2*				
1-6	X			X		72
7-11		X	X		X	24

Table 1. Animals used in different publications.

4.1. Laboratory animals

Three-month-old Finnish female landrace pigs (weight 28-38 kg) were used for the experiments. Animals in each study period were from the same litter. The animals were transported to the National Animal Laboratory Center 4 days prior to each experimental period and were fasted for 12 hours with free access to water prior to the experiment.

4.2. Anesthesia and monitoring

4.2.1. Mode of anesthesia

After premedication with atropine 0.05 mg/kg and azaperone 8 mg/kg intramuscularly an ear vein was cannulated for administration of thiopenthal sodium (5-15 mg/kg i.v.) as induction of general anesthesia. Anesthesia was maintained with infusion of thiopentone (5 mg/kg/h). Pancurone (2-4 mg boluses i.v.) was administered prior to creation of burns and for shivering when needed and fentanyl 30 μg/kg/h during the creation of burn wounds and 5 μg/kg/h thereafter for pain relief.

4.2.2. Mechanical ventilation

Animals were intubated (I) or tracheostomized (II, III and IV) and mechanically ventilated with a volume-controlled ventilator (Servo 900E, Siemens Elema AB, Solna, Sweden) with a tidal volume of 10 ml/kg. Minute volume was adjusted to achieve normocapnia ($paCO_2$ 4.4-5.5 kPa, 34-41 mmHg). Fraction of oxygen in the inspiratory gas (FiO_2) was adjusted to keep arterial partial pressure of O_2 >13.3 kPa (100 mmHg). Positive end expiratory pressure of 5 cmH_2O was maintained throughout the study.

4.2.3. Monitoring

Right carotid artery and internal jugular vein were cannulated for blood pressure and central venous pressure monitoring and blood sampling. Systemic and central venous pressures were recorded with quartz pressure transducers and displayed on a multimodular monitor and recorder (AS3, Datex-Ohmeda, Helsinki, Finland). Continuous information was collected automatically in two minute intervals (Clinisoft, Datex-Ohmeda, Espoo, Finland). All pressure transducers were zeroed to the level of the heart. Heart rate was continuously monitored with electrocardiogram. Haemodynamics were recorded at 15-minute intervals. A urinary catheter was placed through a small incision in the lower part of the abdomen for urinary output measurements. A thermostat controlled operation table heater, warmed fluids and a heat reflector lamp were used in order to maintain normal body temperature (37-39 °C).

4.2.4. Fluid management

Animals received 50 % glucose infusion which was adjusted to maintain normoglycaemia (blood glucose 5-7 mmol/l). Normovolaemia was maintained with Ringer's acetate (Ringersteril, Baxter, Vantaa, Finland) according to CVP 4-7 mmHg. As the total surface area burned was very small, no actual fluid resuscitation due to burn was indicated.

4.3. Laboratory analyses

Arteriolar blood gases and hemoglobin levels were analyzed (ABL 520, Radiometer, Copenhagen, Denmark) in 2-3 hour intervals from the blood drawn from the arterial cannula. Temperature-corrected values were used. The lactate levels were determined with the YSI 2300 STAT plus (YSI Incorporated (Life Sciences), Yellow Springs, Ohio, USA) analysator and the blood sugar levels with the Precision Xtra meter (MediSense, Abbott Laboratories, USA)

4.4. Infliction of thermal injury

After inducing general anesthesia the ventral side of the body of the pig was shaved and washed with chlorhexidine solution (5 mg/ml). The burns were created by using a custom made 4 x 4 cm brass block (weight 530 g) heated to 100 ºC in boiling water (Figure 2). The temperature of the block was measured by having the tip of a digital thermometer (Lutron Thermometer, TM-903, Seoul, Korea) inserted in a drilled hole in the block. By varying the contact time between the block and the animal skin, burns of different depths were obtained. In the first series (Study 1) of publication I contact times of 1, 3, 6, 9 and 12 seconds were used. The contact times were modified from the study by Schomacker et al (1997), where the thicker paraspinal skin of pigs was used. In the second series (Study 2) of publication I and in publications II, III and IV contact times of 1, 3 and 9 seconds were used. Only the weight of the block was used to create the burns and no pressure was applied. In order to avoid variations in burn creation, procedure performance was limited to one person.

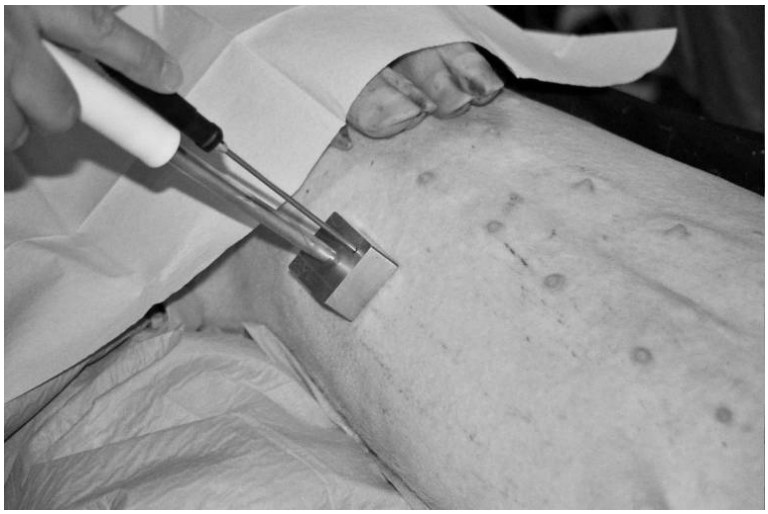


Figure 2. Infliction of thermal injury to ventral pig skin with a brass block heated to 100 °C.

4.5. Histology (I)

In the first series of 6 animals (Study 1) tissue samples with 6 mm punch biopsies were obtained at 2, 24, 48 and 72 hours post burn for histology, one from each quadrant of each burned site extending down to subcutaneous fat to evaluate the time-related progression of burn depth. In the second series of 5 animals (Study 2), which were also used in publications II and IV, biopsies were taken at 24 hours post burn to evaluate the depth of injury. The biopsies from the non-burned control sites of each animal in both series were taken at the same time points identically.

All samples were analyzed by using standard hematoxylin and eosin-stained histological sections of 5 μm-thickness cuts from paraffin embedded samples. A histopathologist analyzed the first 10 samples from the first series together with another pathologist, after which they agreed on the criteria of burn related changes. Evaluation of the samples was done blinded to the details of the sample. After the primary evaluation of all samples, a repeated analysis of 35 randomly selected samples was performed 18 months later by the same histopathologist to test intra-observer reliability by calculating the kappa coefficient as described by Cohen (1960).

Histologically, samples were classified into five anatomical layers: epidermis (level 1), upper 1/3 of the dermis (level 2), middle third of the dermis (level 3), deepest third of the dermis (level 4) and subcutaneous fat (level 5). The location of both thromboses and burn-related histological changes were evaluated respectively. Vascular patency was described as intact vessel walls with normal endothelial cells and no signs of cellular debris or tightly packed erythrocytes. Epidermis was evaluated for burn artifacts (distortion of cell contour) and separation of epidermis from dermis (subepidermal blistering). Dermis was evaluated for histological separation or destruction of different cell layers of hair folliculi and vessel walls, microthrombi and neutrophils. Subcutaneous fat was evaluated for identical adnexal findings as in the dermis, thrombosis and fat necrosis. Burn depth at each evaluation was graded as 1-5 according to the depth of the deepest burn-related histological finding of each sample. In addition, skin thickness was measured from three different points of every sample from the dermo-adipous junction to the surface of the epidermis and presented as the mean of the measured values.

4.6. The microdialysis method (II)

Microdialysis is a sampling technique which enables collection of extracellular fluid in vivo. A tubular semipermeable membrane, the microdialysis probe, connected to afferent and efferent tubes is placed in tissue and perfused. Compounds in the medium surrounding the probe, which are present in higher concentration than in the perfusate and have a suitable molecular weight, diffuse into the dialysis membrane. Concentrations of different substances can be analysed from the dialysate.

After creating the burns, microdialysis was performed by using a CMA/Microdialysis Apparatus (Stockholm, Sweden) with a CMA100 probe (shaft 25 mm, membrane length 10 mm, diameter 0.5 mm, molecular weight cut-off limit 20 kDa) as has been used in histamine studies in humans (Saarinen 2000). A thick needle (Intraflon 2, 16 gauge, ∅ 1.6 mm, Vycon, Ecquen, France) was inserted from one corner of each burned and control area towards the opposite corner, where the tip of the needle was pushed through the skin. The needle was removed and

probe was inserted inside the lumen of the cannula, which was then pulled out from the insertion site leaving the tip of the probe in the middle of the burned area in the dermoadipous plane. Three CMA/Microdialysis Apparatuses and 7 probes were used simultaneously for each burned animal: 1 probe was inserted into each burned area (6/animal) and 1 probe identically to the non-burned control site (Figure 3). The probe was perfused with isotonic saline solution (Ringersteril®) at a flow rate of 3.3 µl/min with a relative recovery of about 35 % for histamine in vitro (Horsmanheimo et al 1994). Eluate fluid for histamine analysis was collected at 1, 2, 6, 12 and 24 hours post injury using a collection time of 30 minutes. Samples for the determination of plasma histamine concentrations were collected from a venous cannula before creating the burn and at the same time as the microdialysis samples.

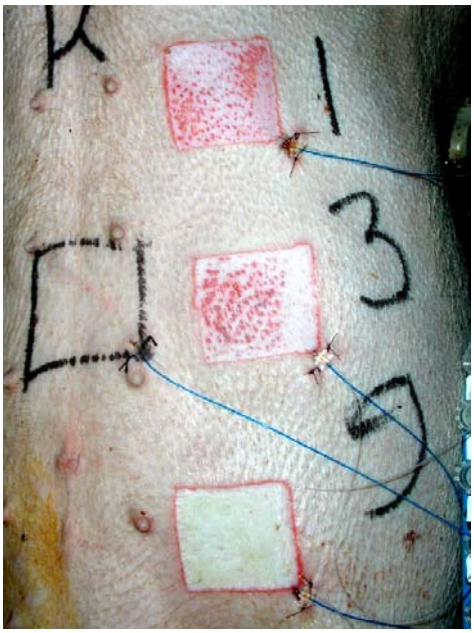


Figure 3. The microdialysis probes inserted in the dermoadipous plane in the 1, 3 and 9 second burn sites and the non-burned control site at 24h postinjury.

4.7. Determination of histamine concentrations (II)

Histamine assay was performed in duplicate of one probe from each site by the radioenzyme method using tritium-labelled S-adenosylmethione (Amersham International, Amersham, UK) and liquid scintillation counting for measurement of the radioactivity as described by Harvima et al (1988). The detection limit of histamine is about 0.5 - 1.0 nM for a 20 µl sample. This sensitivity enables the detection of the histamine content of one single mast cell. At the end of the experiment all burned sites were excised with the microdialysis probe left in place. In order to verify the correct location of the probe, the skin and subcutaneous fat were separated sharply with scissors.

4.8. Dielectric measurements (III)

The dielectric properties of a biological material change with the frequency used in performing the dielectric measurements. The dielectric polarization arises from a physical displacement of charge. The dielectric technique is based on an electronic control unit which generates and transmits high-frequency electromagnetic waves into a coaxial line (Alanen et al 1998). The line is terminated by the open-ended coaxial probe which is manually placed onto the skin. In skin the induced electric field interacts mainly with water molecules in tissue (Foster and Schwan 1989). The extension of the electric field is dependent on the diameter of the circular probe (Lahtinen et al 1997).

The portion of the electromagnetic energy which is not absorbed by tissue water is reflecting. The reflected wave is collected by the same probe (Delfin Technologies Ltd, Kuopio, Finland) as used for wave transmission. From the properties of the reflected wave the dielectric properties of the investigated site are determined (Alanen et al 1998, Stuchly and Stuchly 1980) and analysed by the HP8753C network analyser (Hewlett Packard Co, USA). A high-frequency electromagnetic field is transmitted through the coaxial probe onto the surface of the skin. When performed with 300 MHz, the dielectric measurement specifically gives information about the amount of both bound and free water (= total water) in tissue (Nuutinen et al 1998). Therefore, the relative changes in the measured dielectric constant are also relative changes in the water content of the measured site. Since the dielectric constant of vacuum is 1 (= no water) and that of water 78.5, the values measured on healthy human skin are between these values, typically of the order of 40 (Nuutinen et al 2004). The measurement is non-invasive and instantaneous.

The dielectric measurements were performed from the center of each burn and control site by placing 3 probes (Delfin Technologies Ltd, Kuopio, Finland) of different sizes successively on the injured skin at different time points post burn. The outer diameters of the probes were 5, 15 and 30 mm (Figure 4). The effective measurement depths of these are 0.5, 2.5 and 5.0 mm yielding information from the upper dermis, whole dermis and dermis-subcutaneous fat, respectively (Lahtinen et al 1997). Placing the probe as parallel to the skin as possible and using a gentle contact between the probe and the skin care was taken to both avoid air gap formation between the probe and the skin and, on the other hand, to avoid fluid escape from under the probe (Figure 5). As the dielectric constant is directly related to tissue water content (Schwan 1993-1994) the changes in the dielectric constant represent changes in tissue water content.

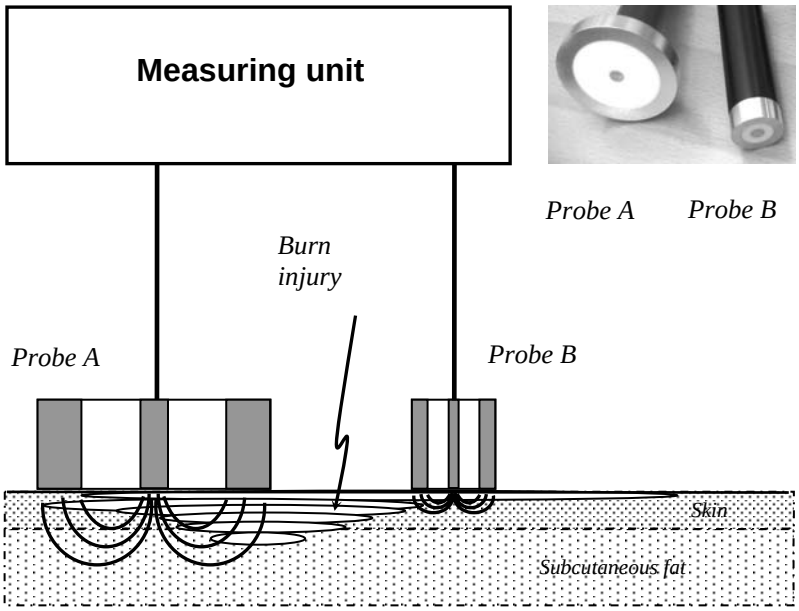


Figure 4. Schematic illustration of the dielectric measurement with burn injury and induced electrical field in subcutaneous fat and skin by a A) bigger (30 mm) or a B) smaller (5 mm), probes not in scale.

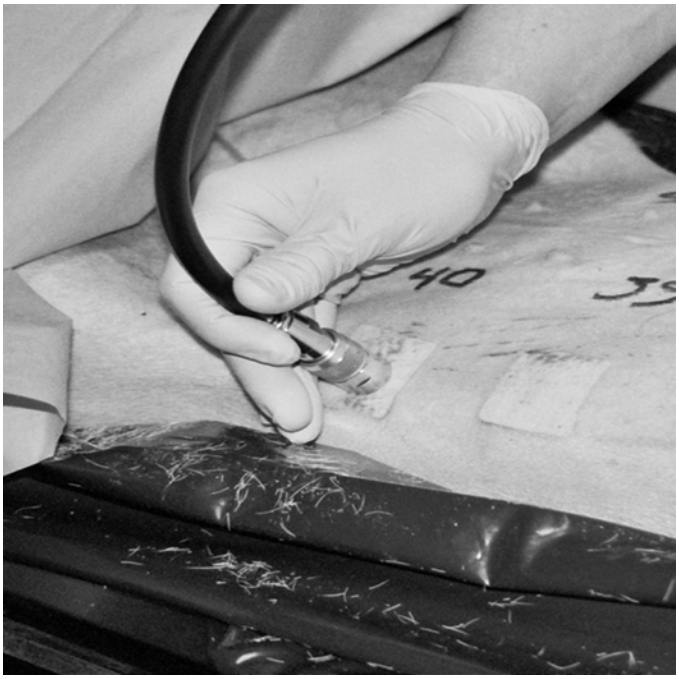


Figure 5. Performance of a dielectric measurement with a 5 mm probe.

4.9. Determination of tissue water content (IV)

The water amount of a material can be determined by lyophilization. The material is first weighted and then frozen and transferred to a drying chamber where it is subjected to a high vacuum. Heat is carefully applied to the material as it supplies the energy necessary for the sublimation of water. An ice crystal is composed of pure water confined in a crystal lattice. Extra thermal energy is needed to break the water free. Broken water diffuses through the solid material and sublimes. The actual force driving water through the dry shell to the surface of the material is the concentration gradient and not the vacuum. The removal of the water that reaches the surface of the material is critical for completion of the drying process. Sublimed water must be removed from the free space of the vacuum chamber by condensers.

In the present study the wet-weighed tissue samples (weight range 0.49-3.62 g) were frozen at -70° C and lyophilized with a Hetosicc CD 52 freeze drying apparatus (Heto, Birkerød, Denmark). Lyophilization was continued until the difference between 2 successive weightings of the tissue sample was smaller than 0.002 g. The water content was calculated as the difference between wet and dry weight. The water content from each tissue sample was also estimated by the dry/wet weight method.

4.10. Determination of the number of labelled red blood cells in tissue samples (IV)

Radiolabelled red cells have been used to determine the blood content in tissue after thermal injury. Blood from the animal is drawn for radiolabelling followed by a re-injection of the labelled blood into the animal. Labelled red cells distribute into blood circulation and the amount of labelled cells can be determined from the radioactivity of tissue samples. Chromium-51 was used by Owen and Farrington (1976) to determine blood content in rat paw after an immersion injury. Other reported labelling methods in burn literature include ^{141}Ce, ^{85}Sr, ^{46}Sc (Ferguson et al 1977), ^{14}C (Wang et al 1995) and ^{99m}Tc (Cetinkale et al 1997).

Two venous blood samples (8 ml each) were collected from each pig prior to infliction of the burns. Both specimens were anticoagulated with 1.5 ml of acid citrate dextrose-A (ACD-A) solution. The samples were centrifuged at 3000 rpm for 10 minutes. Supernatant plasmas and buffy coats were discarded taking care not to remove any red blood cells. Sodium chromate (Cr-51) solution (2 MBq) was slowly added to each tube and the suspensions were incubated for 15 minutes at room temperature with continuous gentle mixing. Labelled cells were washed three times with isotonic saline and re-suspended in the original blood volume with isotonic saline (Lewis and Bayly 1986). Aliquot (0.5 ml) of the labelled red blood cell suspension was taken for

a radioactivity measurement while the rest of the suspension was reinfused in the animal through a vein cannula at 23.5 hours post burn. After 30 minutes (i.e. 24 hours post burn) the animal was sacrificed with an intracardial injection of magnesium sulphate.

Simultaneously with the collection of the blood samples for labelling, one 5 ml blood sample was collected from each pig for the determination of the red blood cell (RBC) count. The RBC count per ml of blood was determined by a Sysmex K-4500 analyser (Sysmex, Kobe, Japan). Since a known volume of blood was drawn for labelling, total number of the labelled and reinfused RBCs could be estimated. After sacrifice the burn sites were excised surgically and the skin was sharply separated from the subcutaneous fat tangentially with scissors. Each tissue sample was again cut vertically in two pieces and placed in a test tube. Hence, from each burn and control site, there were two tissue samples of skin and two samples of subcutaneous fat. Each test tube and tissue sample was weighed separately. The radioactivity of the tissues and the labelled RBC suspensions were counted with a 1480 Wizard gamma counter (Perkin Elmer, Turku, Finland) using a counting time of 30 min. The RBC suspensions were diluted 1:10000 with isotonic saline prior the measurement. Background-corrected counts for the diluted RBC suspensions were used to calculate counts of a single RBC. The number of the labelled RBCs per 1 g of tissue was determined by using background-corrected total counts for tissue samples and calculated counts of a single RBC.

4.11. Statistics

The Helmert contrasts (I) compares the average of the response variable in a time point with the average of the following time point means in sequence. The kappa coefficient was calculated to test the intra-observer reliability and the repeatability of burn creation (I). The non-parametric Wilcoxon signed rank test (2-tailed) was used for statistical analysis using SPSS for Windows (SPSS Inc, Chicago, IL, USA) programme (II, III, IV). The correlation coefficient (R) was calculated with the Excel for Windows programme (II). A Bonferroni correction was used and the receiver operating characteristic (ROC) curve analysis with the corresponding area under the curve (AUC) was performed and positive and negative predictive values (PPV, NPV) calculated with the MedCalc Version 7.4.1.0 for Windows programme (III). Statistical significance was defined as $p < 0.05$ (I-IV).

5. RESULTS

5.1. Histology (I)

5.1.1. Study 1

The burn depths at different time points in this 72-hour-follow-up are presented in Figure 6. At all time points the 1 second burns were the most superficial and graded as superficial dermal burns (level 2). The 3 second burns were more superficial than the 6, 9 and 12 second burns ($p = 0.001$). Similarly, the 6 second burns were more superficial than the 9 and 12 second burns ($p = 0.006$). No difference was seen between the 9 and 12 second burns ($p = 0.104$). Also, there was a time-related progression of burn depth during the study (Figure 6) except in the 1 second burns, where there was no progression of burn depth. In the other burn sites the depth of the injury was more superficial at two hours post burn compared to any later time point ($p < 0.0005$) and also at 24 hours compared to later time points ($p = 0.015$), but there was no difference between 48 and 72 hours post burn ($p = 0.397$). Accordingly, progression of burn depth was histologically evident until 48 hours post burn.

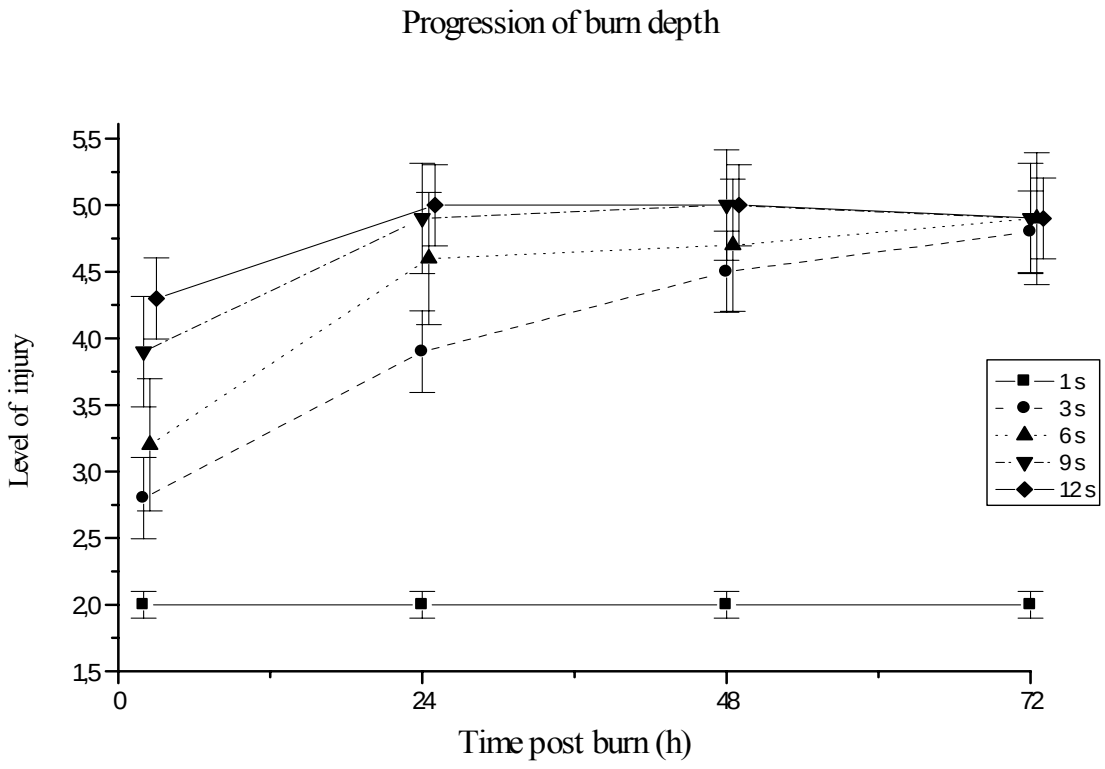


Figure 6. Progression of histological depth (± 1 SE) of all burn sites during follow-up. Anatomic level of thermal injury is graded 1-5.

Histologically at 24 hours post injury the 1 second contacts lead to a superficial dermal injury (level 2), the 3 second burn to a partial thickness injury (level 3-4) and the 9 second burn to a full thickness (level 5) injury. Accordingly, these contact times and the follow-up time of 24 hours were chosen for study 2. The main histological findings are presented in Figures 7 a-c.

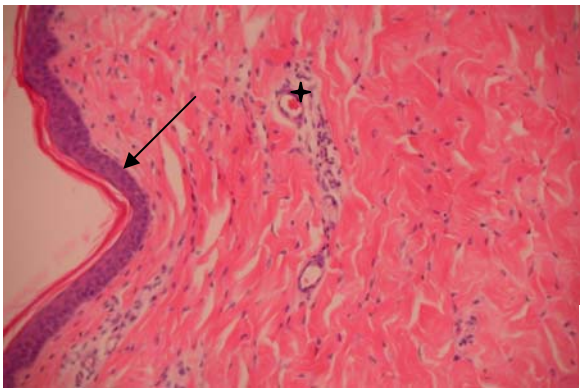


Figure 7a. Normal pig skin from non-burned control area with intact epidermal layer (arrow) and the patent vessel (asterix). 10X

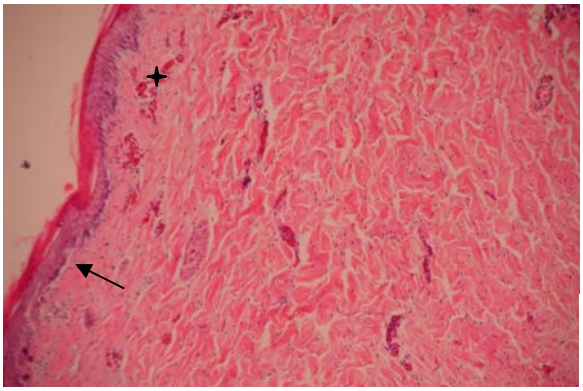


Figure 7b. Subepidermal blistering (arrow) and hyperemia (asterix) in a 12 s burn 2 hours post burn. (10X)

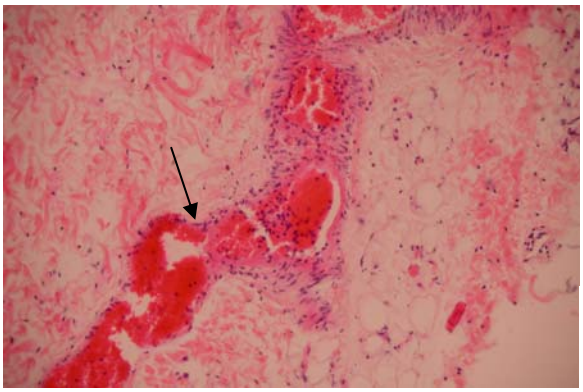


Figure 7c. Thrombosis (arrow) within the lumen of a vessel in the dermo-adipous junction. Also neutrophil infiltration is present. (20X)

5.1.2. Skin thickness (I)

The skin thickness of the non-burned control site remained unchanged (1.6 mm, range 1.5 - 1.73 mm) throughout the study. The skin thickness in the 1 second burns was greater compared to both the control site ($p = 0.005$ at all time points) and to the 3, 6, 9 and 12 second burn sites ($p = 0.005$-0.012) throughout the study period. The skin thickness in the 3, 6, 9 and 12 s burns was greater than the skin in the control site at all times ($p = 0.005$-0.037) except the 3 s burn at 2 hours ($p = 0.285$) and 72 hours ($p = 0.92$) and the 12 s at 72 hours ($p = 0.767$). There were no differences between the 3, 6, 9 and 12 second sites.

5.1.3. Study 2

The 3 second burns were significantly deeper than the 1 second burns ($p = 0.004$) and the 9 second burns were deeper than both the 1 second ($p = 0.006$) and the 3 second burns ($p = 0.005$). The mean burn depths of different contact times are presented in Figure 8.

The kappa coefficient for intra-observer variation between two repeated analyses of 35 histological samples by the same pathologist in Study 1 was 0.83. The repeatability of burn depth creation between Studies 1 and 2 yielded a kappa coefficient 0.92.

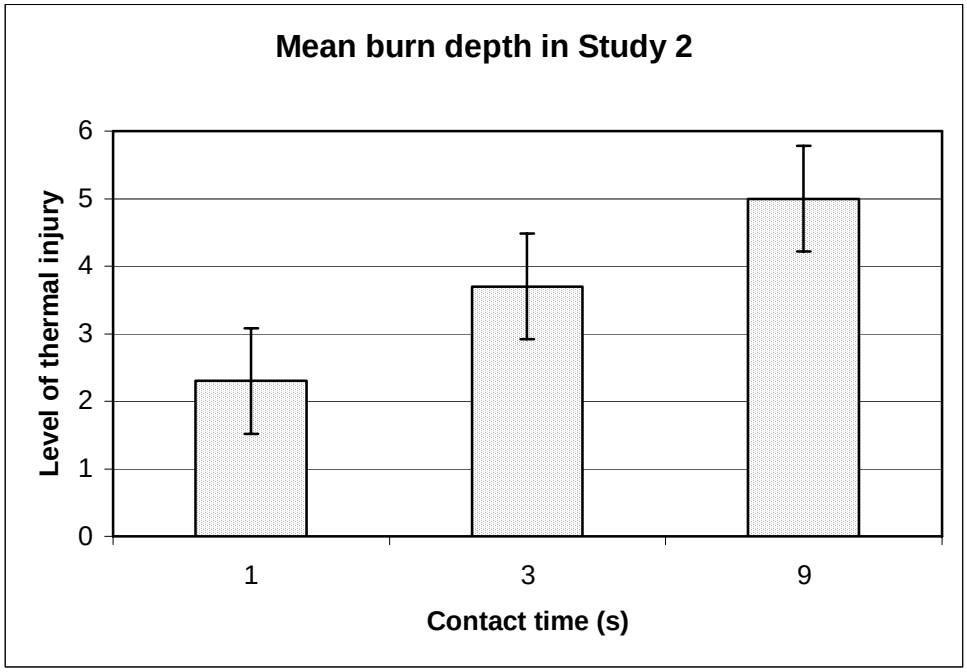


Figure 8. Mean histological depth (± 1 SE) of thermal injury in Study 2.

5.2. Histamine measurements (II)

5.2.1. Histamine concentrations in tissue

The histamine concentrations in the microdialysis eluate and in plasma are presented in Figure 9. A good correlation was found between the contact time and the tissue histamine concentration at 1 and 2 h post burn with correlation coefficients (R) of 0.999 and 0.927, respectively (Figure 10). The full thickness 9 second (9S) burn induced higher histamine concentrations in the extracellular fluid than the 1 second (1S) and 3 second (3S) burn sites at 1 hour (p=0.003 and 0.019, respectively), at 2 hours (p = 0.01 and p = 0.003, respectively) and at 6 hours (p = 0.03 and 0.002, respectively) post burn. There were no differences between any burn sites at 12 and 24 hours. At all time points all burn sites had higher histamine concentrations than the control sites (p = 0.012-0.034) except the 3S at 6 hours.

There was a significant decrease in histamine concentrations between 2 and 6 hours post burn in all burn sites (1S and 3S, p = 0.007; 9S, p = 0.008). No changes were found between 6 and 12 hours. However, between 12 and 24 hours post injury there was a significant increase in tissue histamine concentrations in all burn sites (1S and 3S, p = 0.007; 9S, p = 0.028). The highest concentration of histamine in the end of the study was in the 1S and 3S sites and the lowest concentration in the 9S, although the differences between different burn sites at this time were not statistically significant.

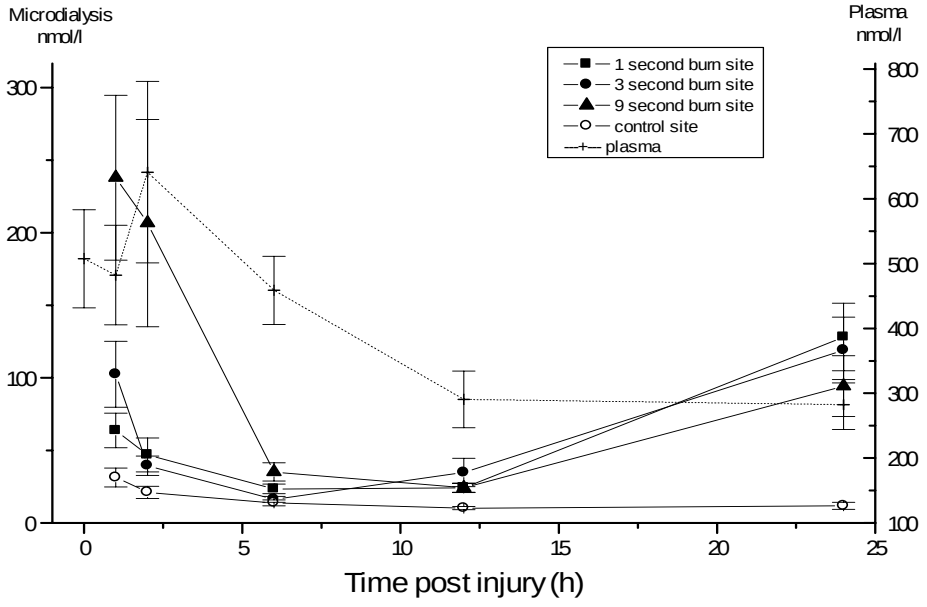


Figure 9. Histamine concentrations (median ± quartiles) in the microdialysis eluate and plasma at different time points.

5.2.2. Histamine concentrations in plasma

Thermal injury caused an increase in plasma histamine concentration until 2 hours post injury (Figure 9). Thereafter, the histamine concentrations decreased reaching a plateau at 12 hours post injury. The late increase in tissue histamine levels was not seen in plasma.

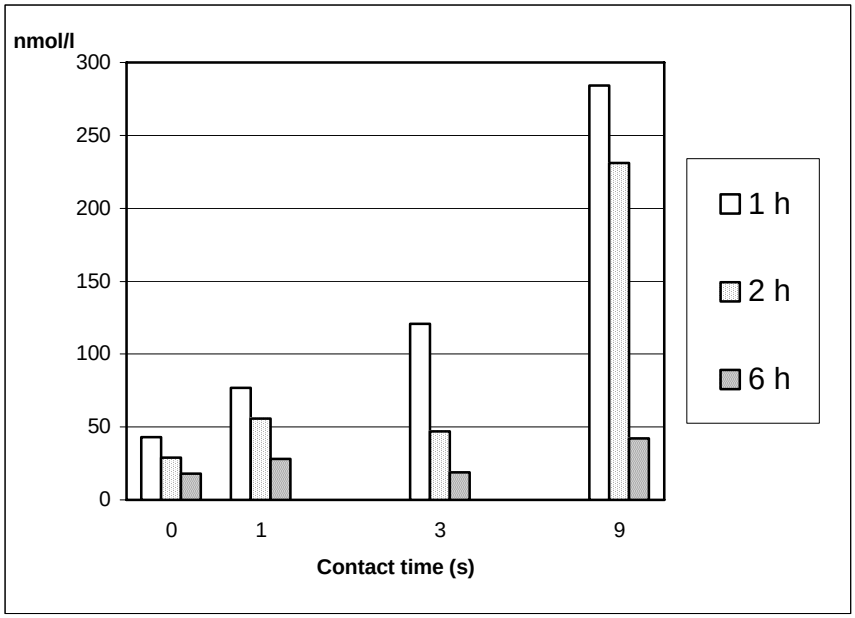


Figure 10. Burn duration -related histamine concentration in tissue presented as a function of burn duration at 1, 2 and 6 hours post injury. The values at 0 seconds represent the non-burned control site.

5.3. Dielectric measurements (III)

5.3.1. Edema formation

In the superficial (1S) burn site, there was no difference in the water content in the upper dermis (Fig. 11A) compared to the control site. In the whole dermis (Fig. 11B), however, the water content was increased at 8 h compared to the control site. There was a significant increase in tissue water content when the subcutaneous fat was also included in the measurements (Fig. 11C). The partial thickness (3S) burns (Fig. 12A-C) resulted in similar findings as the superficial burns. On the other hand, the full thickness burns showed lower water content in the upper dermis (Fig. 13A) throughout the follow-up period although the reduction was significant only at 24 hours. There were no differences compared to the control site in whole dermis (Fig. 13B), but the tissue water content was very high again in the subcutaneous fat (Fig. 13C).

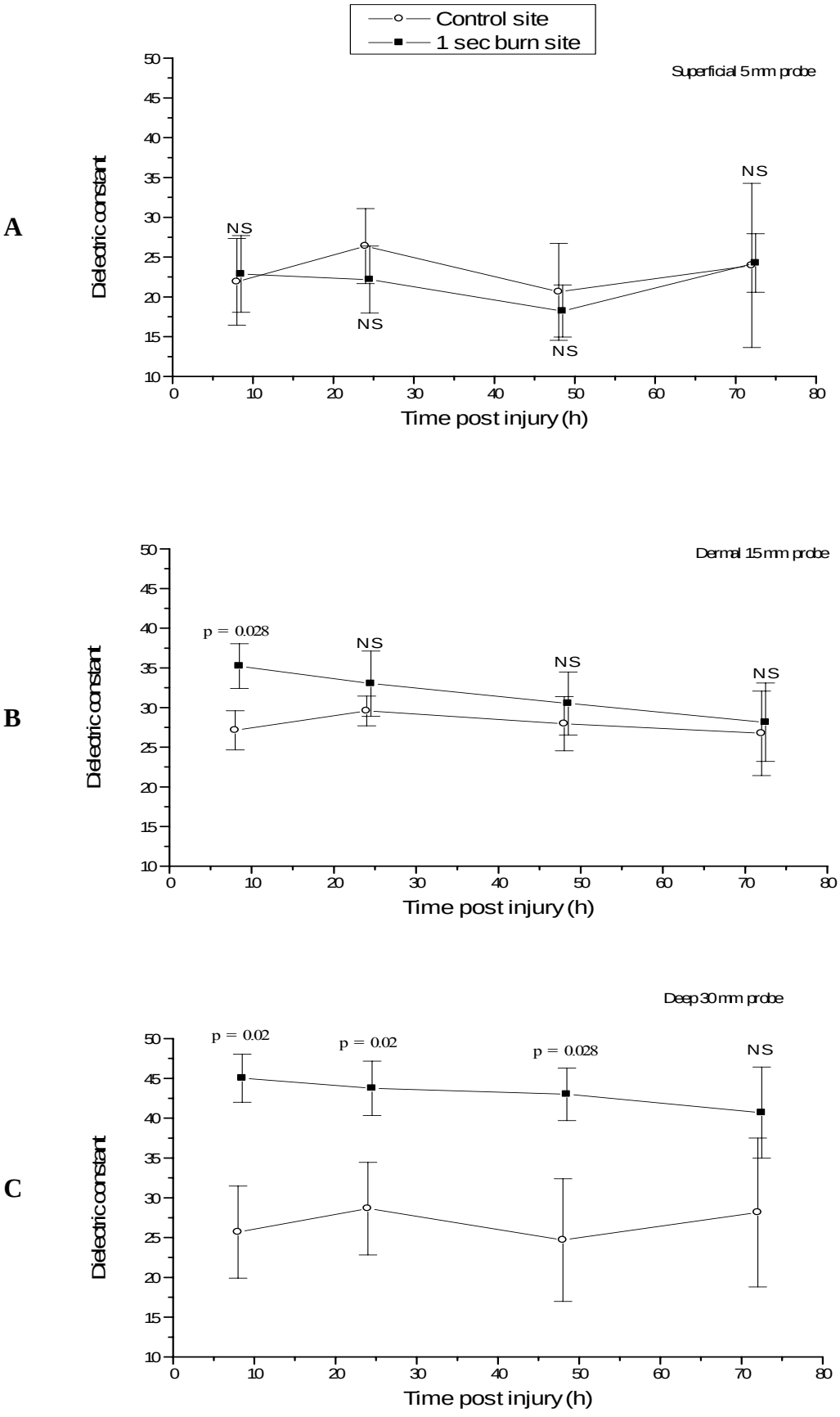


Figure 11. The mean (± SD) dielectric constants in the 1 second burns and the control site measured with the (A) 5 mm, (B) 15 mm and (C) 30 mm probes with the respective p-values.

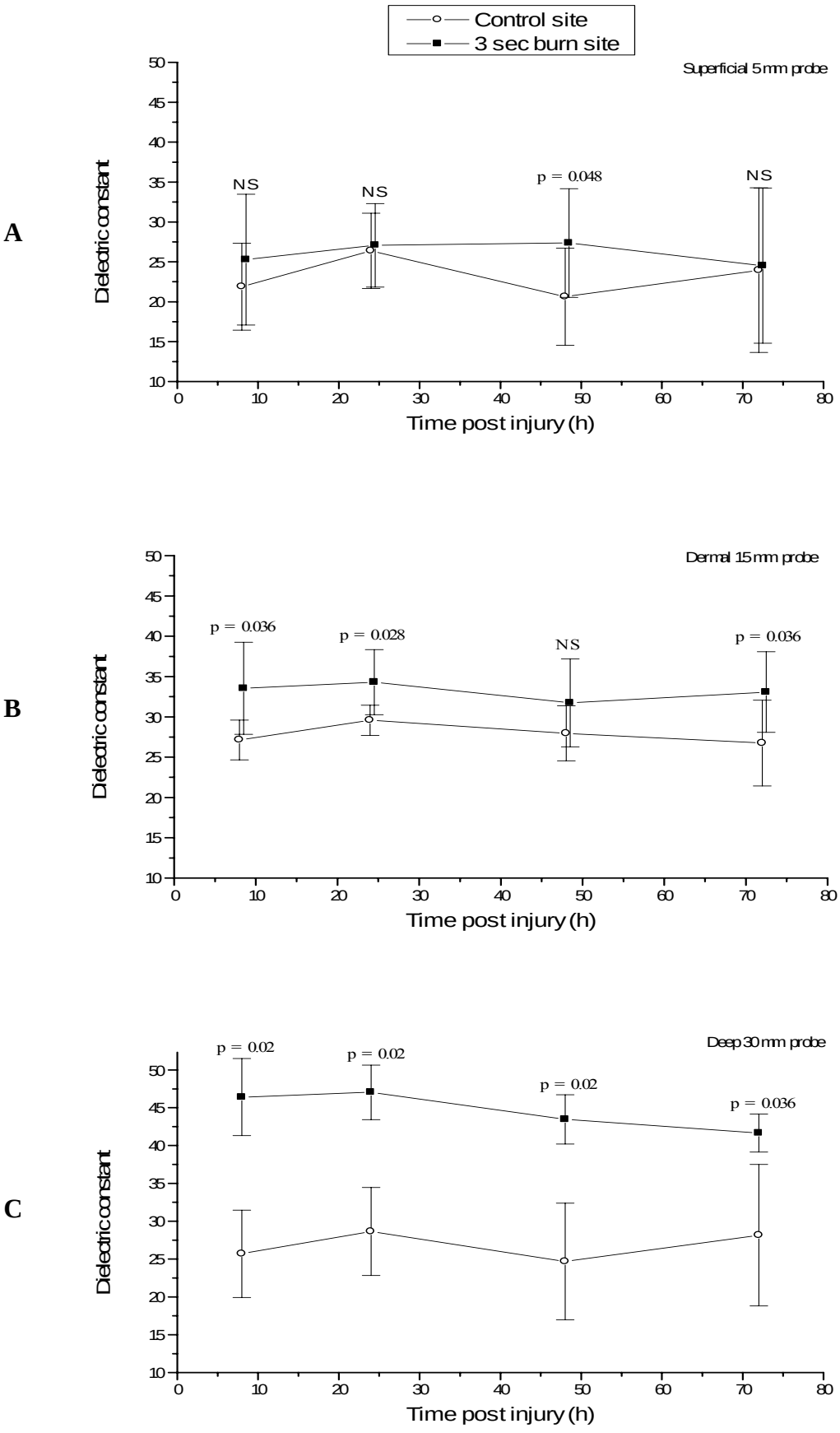


Figure 12. The mean (± SD) dielectric constants in the 3 second burns and the control site measured with the (A) 5 mm, (B) 15 mm and (C) 30 mm probes with the respective p-values.

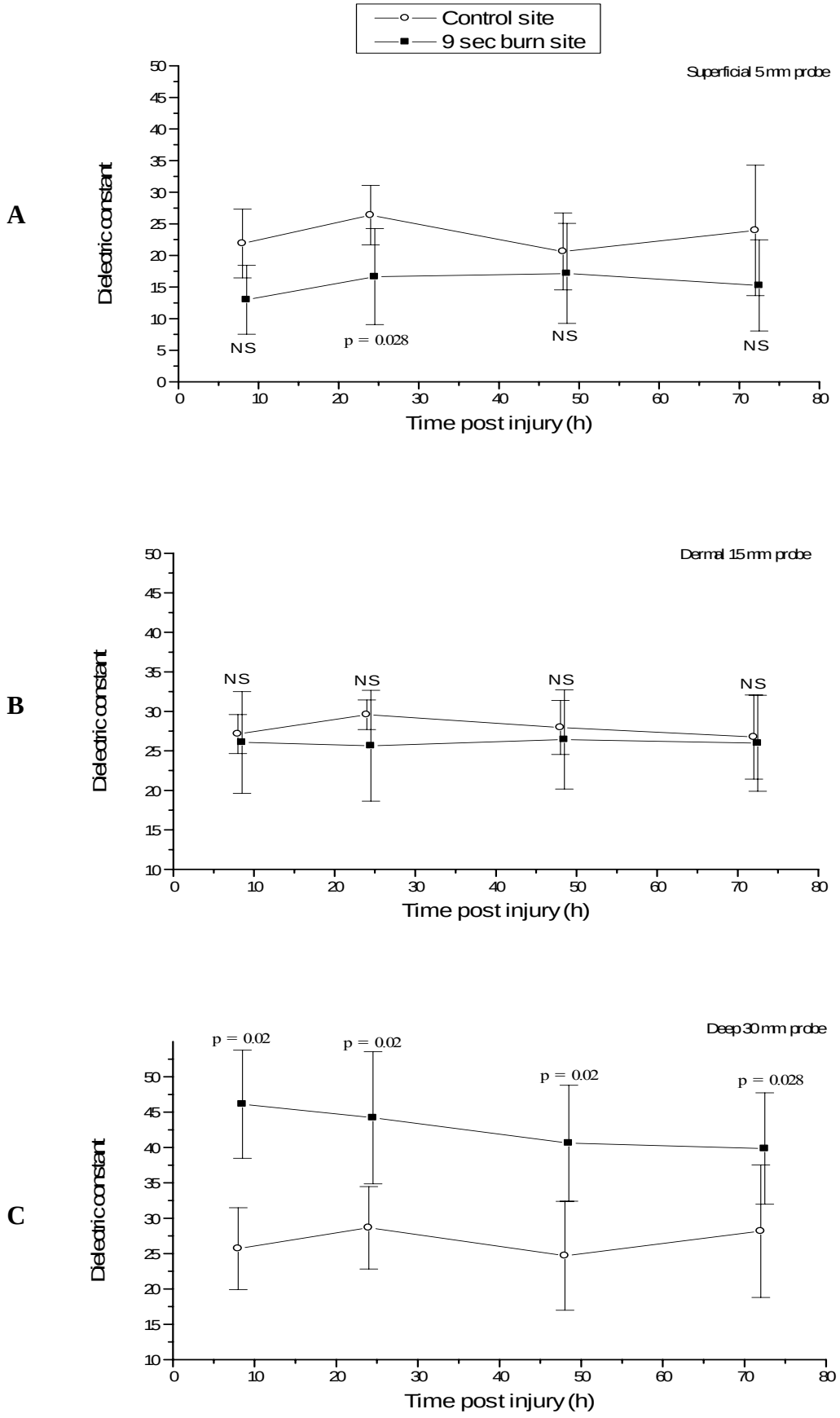


Figure 13. The mean (± SD) dielectric constants in the 9 second burns and the control site measured with the (A) 5 mm, (B) 15 mm and (C) 30 mm probes with the respective p-values.

5.3.2. Differentiation of partial (3S) and full thickness (9S) burns

5.3.2.1. Superficial (5 mm) probe

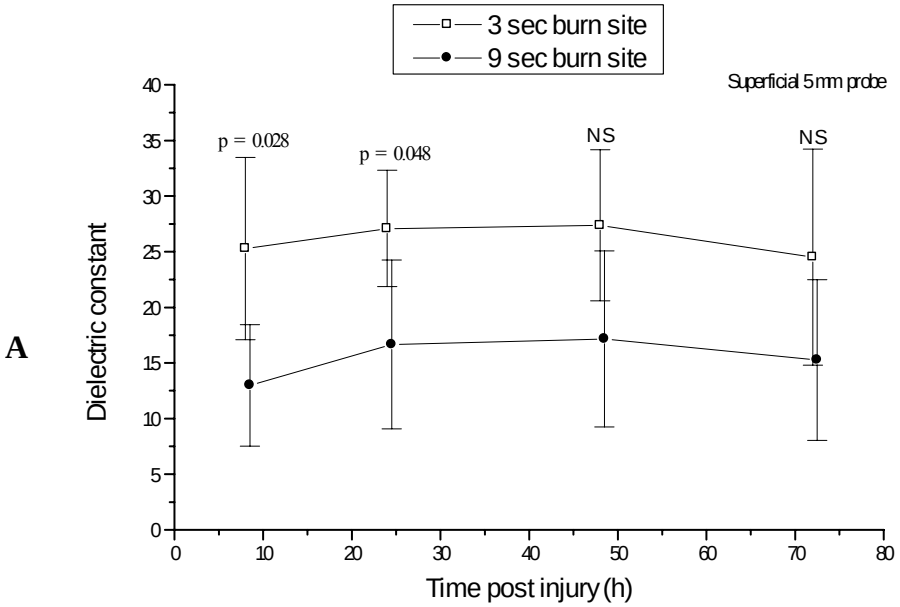
The tissue water content in superficial dermis in the 9 second burn sites was significantly lower ($p = 0.028 – 0.048$) than in the 3 second burn sites during the first 24 hours (Fig. 14A) and lower than in the control site ($p = 0.028$) at 24 hours post injury (Fig. 13A).

5.3.2.2. Dermal (15 mm) probe

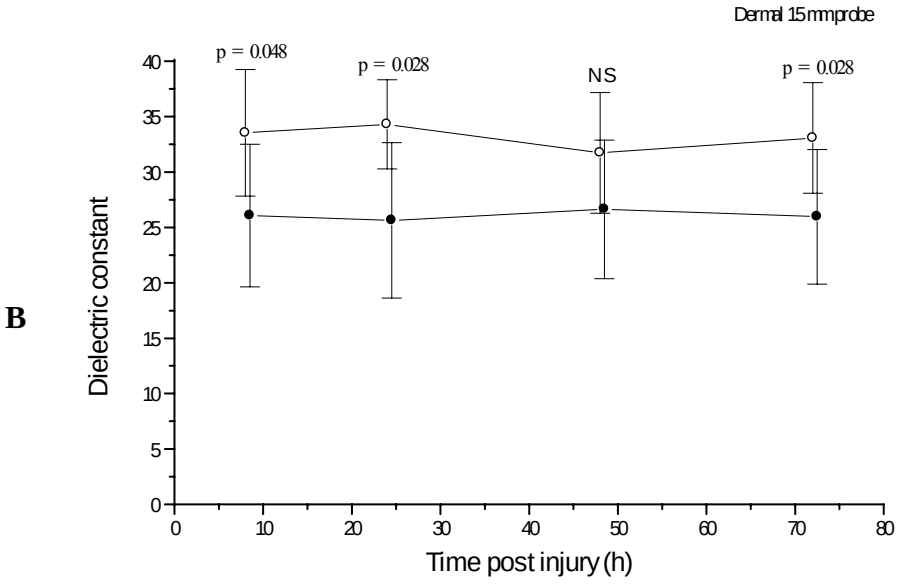
The 9S burns had lower tissue water content in whole dermis (Fig. 14B) than the 3S burns at 8, 24 and 72 hours post injury ($p = 0.028 – 0.048$).

5.3.2.3. Deep (30 mm) probe

The tissue water content at all burn sites in subcutaneous fat (Fig. 11C, 12C and 13C) were significantly higher than at the control site ($p = 0.02$-0.036) except in the 1S burn site at 72 hours post injury. There were no differences between burn sites.



A



B

Figure 14. Comparison of the mean (± SD) dielectric constants in the 3 and 9 second burn sites measured with the (A) 5 mm and (B) 15 mm probes with the respective p-values

5.3.3. Receiver operating characteristic (ROC) curve analysis, area under the curve (AUC) and positive (PPV) and negative (NPV) predictive values

The ROC curve analysis was performed in order to find the threshold values for the dielectric measurements distinguishing the 3S and the 9S burns at different times after injury. The results of the ROC analysis are presented in Table 2. A deep burn was classified as a positive finding. At 8 hours the superficial 5 mm probe had an excellent specificity (100 %) and PPV (100 %) and a good AUC (0.92). The AUC decreased in time as did the specificity. On the other hand, the ability of the superficial dielectric measurements to find true positive findings (=sensitivity) increased towards the end of the study. The AUC values in the dermal (15 mm) probe measurements were lower than in the measurements with the superficial probe. The positive predictive values increased between 24-72 hours. The NPV values were lower than the PPV values. An example of a roc curve is presented in Figure 15.

Probe	Time (h)	DC	Sensitivity (%)	Specificity (%)	AUC	PPV (%)	NPV (%)
5mm	8	13	70	100	0.92	100	59
(superficial)	24	19	70	100	0.88	100	59
	48	20	80	90	0.85	99	33
	72	19	90	80	0.82	95	67
15 mm	8	29	80	80	0.82	94	50
(dermal)	24	26	70	100	0.88	89	10
	48	28	80	80	0.78	94	50
	72	28	80	90	0.86	97	53

DC = dielectric constant resulting in the best cutting point between 3 and 9 second burns
AUC = area under the curve
PPV = positive predictive value
NPV = negative predictive value

Table 2. Results of the ROC curve analysis. The dielectric values giving the highest accuracy of the method to distinguish the 3 second burns from the 9 second burns with the 5 and 15 mm probes are presented together with the respective values of AUC, PPV and NPV.

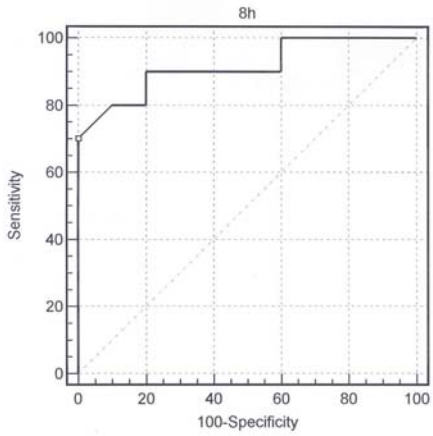


Figure 15. The receiver operating characteristic curve (5 mm probe) at 8 hours post injury. Cut-off point (value = 13) is marked with ▫ with a 100 % specificity and 70 % sensitivity.

5.4. The amount of labelled red cells and water in tissue (IV)

5.4.1. Number of radioactively labelled red cells (IV)

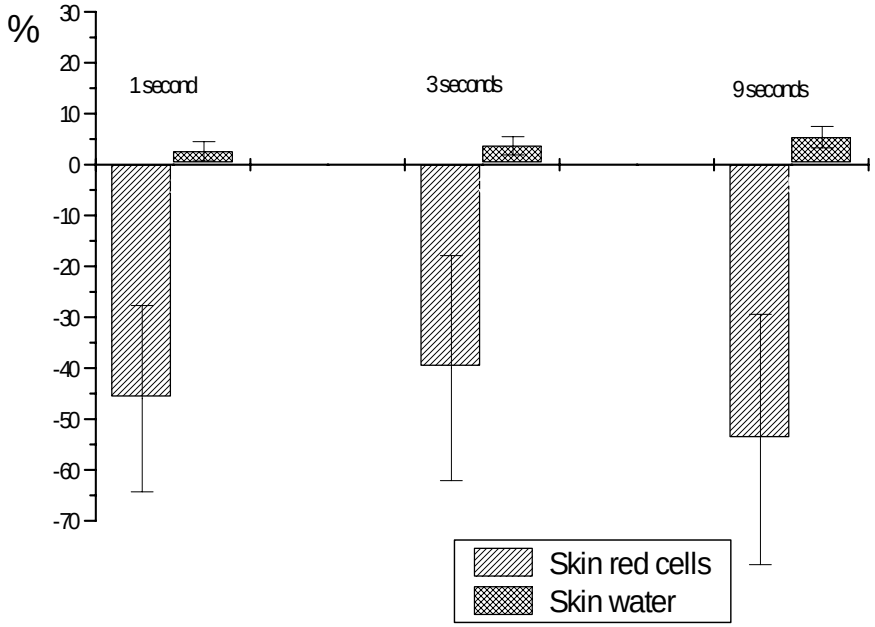
The superficial (1S), partial (3S) and full thickness (9S) burns caused a 46 %, 40 % and 54 % decrease, respectively, in the number of labelled red cells (LRC) in skin (Fig. 16A) at 24 hours post injury ($p = 0.028$ in all). The 1S and 3S burns caused an insignificant increase of 15 % and 12 % in LRC in subcutaneous fat (Fig. 16B) compared to the control site. The 9S burns caused a 19 % decrease of LRC compared to the control site (CS) ($p = 0.028$) and was significantly lower than the 1S ($p = 0.01$). The difference between the 3S and 9S burn sites was non-significant ($p = 0.091$). In the 1S and 9S burn sites the number of LRC in fat was higher than in skin ($p = 0.003$ and $p = 0.010$, respectively).

5.4.2. Water amount in skin and subcutaneous fat (IV)

In the unburned control site the content of water in skin was 72.3 % and in the subcutaneous fat 36.6 %. While the slight increase in the water content in the burned skin was insignificant (Fig. 16A), the water content in fat (Fig. 16B) increased 64 %, 77 % and 67 % in the 1S, 3S and 9S burn sites, respectively, compared to CS ($p = 0.028$ in all). The water content in the 3S burn site was higher than in the 1S ($p = 0.003$) and in the 9S ($p = 0.033$) burn sites.

The water amount can be presented also as wet-to-dry (W/D) ratio where the ratio between the wet and dry weight of a tissue sample is calculated. The relative W/D values (non-burned control site = 1) at different sites were quite identical with the results of the dielectric measurements (Table 3.) especially in fat (unpublished data).

A



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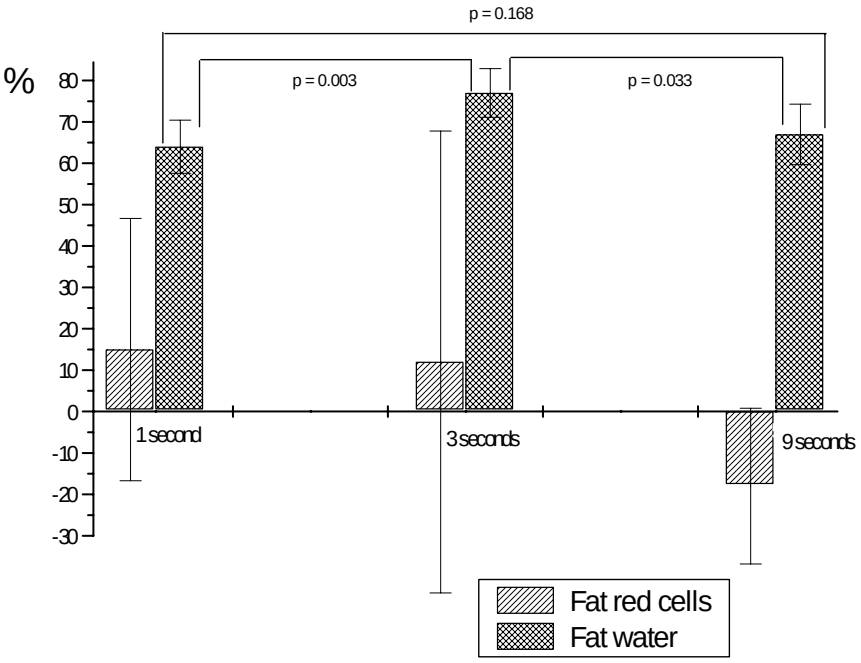

Figure 16. The changes in the amount of labelled red cells and tissue water in (A) skin and (B) subcutaneous fat at different burn sites. Values express the mean (±sd) percentual changes compared to the non-burned control site.

	site	wet to dry ratio	dielectric constant
skin	control	1	1
	1S	1.07	1.11
	3S	1.11	1.15
	9S	1.17	0.86
fat	control	1	1
	1S	1.61	1.53
	3S	1.85	1.64
	9S	1.68	1.54

Table 3. The relative values (mean) of the wet to dry ratio and dielectric constants in skin and subcutaneous fat in different burn and control sites at 24 hours post burn measured with the 15 (skin) and 30 (fat) mm probes. The values of the control sites are expressed as 1.

6. DISCUSSION

Depth of injury dictates the treatment of burn wounds. The most superficial burns heal conservatively without scarring whereas full thickness burns require excising and skin grafting because the epidermal elements needed for conservative wound healing are destroyed. Previously, full thickness burns were allowed to demarcate and operative treatment was scheduled only after the separation of dead tissue was apparent. Nowadays, the aim is for early surgical intervention because the final functional and aesthetic outcome is better and it has proven to be economically beneficial, too (Munster et al 1994). Early burn depth determination is needed to enable early surgical intervention. The goal is the recognize burns which will not heal within 3 weeks because there is an enhanced risk of scarring beyond that time point. Several methods have been reported to determine burn depth. However, none of the methods of early burn depth determination are based on numerical evaluation of burn-related edema, which is the basic local event after thermal injury. The aim of this study therefore, was to create a reproducible burn model to examine novel techniques and see wheter these enable early burn depth determination.

6.1. Study methodology and subjects

These experimental burn studies were performed by using farm swine as laboratory animals. Rats and mice are the most commonly used animals in burn wound research, mostly for economical reasons. However, they differ from humans tremendously in size, life span, overall metabolic rate, nature of their diet and nutritional requirements (Douglas 1972). Also, they, as other rodents, have much thinner skin than human and their wounds heal mostly by contraction due to the anatomic entity panniculus carnosus which is not found in humans or pigs. The different wound healing mechanisms become troublesome especially if conservative healing times are being evaluated. Farm swine was chosen in this study series because its skin has several anatomical similarities to human skin (Brans et al 1994). The skin of pig and human is almost indistinguishable by physiological, anatomical and physical resemblance, with humans having slightly thinner skin (Douglas 1972). In experimental burn research it is also important to choose a sufficiently large animal whose flat surfaces permit creation of uniform burns (Singer et al 2000). The thick dorsal paraspinal skin of pigs has previously been used in burn wound research (Brans et al 1994, Nanney et al 1996). In this study, however, the thinner ventral side of the body was used due to its close resemblance of human skin especially in skin thickness. It is likely that when local events in skin are examined in burn experiments, an animal whose skin resembles most closely to that of human would be ideal. On the other hand, other animals could be used, when burn-related systemic reactions are being evaluated.

General anesthesia and invasive monitoring was used throughout each experiment. The animals were kept on the respirator in order to standardize the general conditions as thoroughly as possible. The longest experiments lasted for 72 hours, which seemed to be quite a maximal time for a pig to be on the respirator. Longer follow-up time of the burn wounds would have required both wakening up the animals and a demandingly meticulous wound care in order to avoid wound infections. That might have resulted in variations between animals considering wound healing. A follow-up time of three weeks would have shown, however, which burns would actually heal within 3 weeks, a time which is considered a milestone for conservative treatment in partial thickness burns (Heimbach et al 1984).

The burns were rather small in size so no burn-related systematic effects of the burns were noted in the invasive haemodynamic monitoring, laboratory analyses or urine output. The mean area burned per animal was 1.2 – 2.2 % of the total body surface area when a considerably larger burn is needed to cause systematic effects (Arturson and Jonsson 1979). Therefore, it can be concluded, that all changes in the variables measured at the burn sites represent local and not systematic reactions.

The number of animals was rather limited in this study. This was partly due to economical reasons. On the other hand, the purpose of publication I was to create a burn model for further research purposes. In a large animal, like a farm swine, several burns could be created per animal thus reducing the number of animals needed. In publications II and III the aim was to examine whether the microdialysis technique and the dielectric measurements were applicable in burn wound research and in publication IV to confirm the findings of increased tissue water found in publication III. As the results provided evidence of achieved goals the number of animals was not increased.

6.2. Histological diagnosis of burn depth

Histology has been considered the gold standard for burn depth determination although it has several concerns (Heimbach et al 1992). The timing of the biopsy is controversial as burn depth is known to progress during the first few days (Hinshaw 1961, Converse et al 1965, Zawacki 1974a, Kaufman et al 1990, Brans et al 1994, Nanney et al 1996, Schomacker et al 1997, Watts et al 2001). Burn sites might not be uniform in depth making the site of the biopsy questionable. Biopsy leaves a permanent scar, is expensive and time consuming and requires an experienced histopathologist to make the analysis. Also, the subjective nature of the evaluation is troublesome. The only reliable criterion differentiating a partial thickness burn from a full thickness burn is the presence or absence of surviving viable epithelial elements in the injured area (Hinshaw 1961).

The nature of the skin appendages can be the most informative aspect to the observer. In addition, it is very important to evaluate blood vessels and their contents in determining the viability of burned tissue (Kahn et al 1979, Watts et al 2001). Blood vessels are found in every histological section from skin, follicles are few. The identification of the depth of vascular or follicular necrosis is easiest to achieve and may be of more clinical relevance than other elements (Singer et al 2000). In the present study all these elements were separately evaluated histologically in addition to cell distortion and epidermal separation from the dermis, epidermal blistering, starting from the basal cell layer, and fat cell necrosis.

Timing of the histological evaluation of burn depth varies greatly in different studies ranging from immediately after inflicting the burn (Ho-Asjoe et al 1999) to 21 days post burn (Behounkova-Houskova and Moserova 1979). In the present study, the progression of histological burn depth was initially evaluated repeatedly in 6 animals with a total of 50 burn sites of 5 different depths during a 72-hour period. Of these, the 1, 3 and 9 second contact burns created with a brass block heated to 100 °C resulted in superficial, partial thickness and full thickness burns at 24 hours post injury, respectively, and that time point was hence chosen for the further studies. The repeatability of burn depth creation between the two studies in the 1, 3 and 9 second burns yielded a kappa coefficient of 0.92.

During the initial 72-hour study there was a clear progression of burn depth until 48 hours. This is in accordance of the previous studies mentioned earlier. However, there was no progression in the most superficial 1 second burns, which has also been reported earlier (Watts et al 2001). Surprisingly, in the end of the study the 3 second burn sites lead to a histological burn depth which was graded as a level 5 burn extending down to the subcutaneous fat. This might relate to the fact that the pattern of vascularization in pig skin displays a lower, mid-dermal and a sub-epidermal vascular network. It differs from human in that the subepidermal network is less dense (Meyer et al 1978). This would enable the superficial or partial thickness burn to progress in depth more easily. These burns, however, might have healed conservatively like partial thickness burns, but a three week follow-up time would have been needed to confirm that.

6.3. Concentrations of histamine in tissue and plasma and the microdialysis method

Histamine has been considered an important local mediator in thermal injury. Histamine in skin is mostly stored in mast cells. Histological examination shows disruption of histamine granules in mast cells of burned skin (Sanyal 1962). The early rise in blood histamine in cutaneous burns is primarily due to the release of histamine from the skin (Suzuki et al 1971, Yurt and Pruitt 1986). Interstitial histamine in the dermis has been found to represent specifically the local tissue

concentration (Krogstad and Roupe 2001). In moderate burns the rise in tissue histamine concentration is delayed but is immediate in severe injury (Horakova and Beaven 1974). Tissue histamine concentration has also been found to decrease more rapidly in severe injuries compared to moderate ones (Horakova and Beaven 1974). The release of histamine closely parallels the development of edema and once edema is fully developed no further release of histamine occurs (Horakova and Beaven 1974).

The original idea of sampling the extracellular fluid for analysis was described by Bito et al (1966) and the later technically improved perfusion system was presented by Ungerstedt (1984). Among the first studies with humans, the histamine concentration was measured from the ventral forearm (Anderson et al 1992). A rise in skin histamine has been found due to insertion of the probe. Therefore, an equilibrium time of 40 (Anderson et al 1992) to 90 (Groth 1996) minutes in humans and 30 minutes in rats (Groth 1996) following probe insertion is suggested where skin provocation is performed. Histamine concentrations have shown to be independent of probe depth in skin (Andersson et al 1996).

Several factors affect the dialysis recovery of substances (Groth 1996). Increasing perfusion rate compromises recovery but results in increasing sample volume. Recovery is directly proportional to the area of the membrane and increases at higher temperature. There is an inverse correlation between recovery and molecular weight. The pore size of the membrane determines which compounds are allowed to pass the membrane. The perfusion medium should resemble and ideally be identical to the medium surrounding the probe.

In the present study all burn sites induced an increase in tissue histamine concentrations found in the analysis of the microdialysis eluate collected from the burned sites. The initial increase was most profound in the full thickness burns, where the histamine concentration was 2.3 and 3.7 times higher than in the superficial and partial thickness burns, respectively. The rapid increase is in correlation with the earlier findings (Suzuki et al 1971, Horakova and Beaven, 1974, Markley et al 1975, Devereux et al 1975, Yurt and Pruitt 1986, Santos et al 2000). This is most probably due to the rupturing of the histamine granules in mast cells which in normal skin are of greatest density in the superficial dermis. It is likely, that deep burns destroy more mast cells than superficial ones resulting in higher tissue histamine concentrations in the early stage after burning. The initial tissue histamine concentrations were elevated also in the non-burned control sites indicating either a systemic effect caused by the induction of general anaesthesia or adjacent burns or more likely, a reaction caused by the insertion of the microdialysis probe as is found in human studies (Saarinen 2000).

The decrease of tissue histamine concentrations until 12 hours post injury was followed by a secondary increase seen at 24 hours post burn. The histamine concentrations at this time point

were marginally lower in the full thickness burns than in the partial thickness and superficial burns, but the difference was not statistically significant. There are some possibilities explaining this finding. First, the higher tissue histamine concentration seen in partial thickness burns could be partly due to the histamine from plasma instead of local mast cells since the blood vessels in the deep dermis are not totally obliterated at 24 h post burn due to the progression of burn depth until 48 h. The permeability disorder would hence allow enhanced histamine leakage from the blood vessels. Secondly, the subsiding edema might lead to increased concentration of substances in tissue. Thirdly, when the initial vasoconstriction subsides (DeCamara et al 1982), the increasing blood flow may bring other mediators to the wound site, like anaphylatoxins (Siraganian 1994) triggering the release of histamine locally from the originally surviving mast cells. Partially damaged mast cells liberate histamine, but are able to survive and make new histamine granules. Furthermore, the potential release of neuropeptides, like substance P, from the damaged nerve endings are known factors involved in the delayed histamine deliberation in burns (Dunnick et al 1996). This neuropeptide induces vasodilatation and vascular permeability by stimulating endothelial cells to round up, vascular smooth cells to relax, and mast cells to release histamine. The use of an aminosteroid-type muscle relaxant pancurone might be a confounding factor in our study as it is known to induce rapid histamine release (Koppert et al 2001). However, in the present study it was administered only for shivering, when needed.

The highest concentration of histamine in plasma was seen an hour later than the highest concentration in tissue**.** This supports the previous suggestion of the early rise in blood histamine being primarily due to the release of histamine from the skin (Suzuki et al 1971). The plasma histamine concentration was high already in pre burn samples. This must relate to the induction of general anaesthesia, placing the invasive monitoring cannulae and manipulation of the animal. Unfortunately no baseline plasma histamine concentrations were determined. This would have required stabilizing the animal for several hours after inducing general anaesthesia and placing the probes and multiple sample collection during several hours. The histamine concentration in plasma decreased until 12 hours post burn and stabilized after that. Accordingly, no late increase in histamine levels in plasma was seen as was in tissue. Hence, the late rise in tissue histamine concentration is rather a local event than a systemic one.

Tissue histamine concentrations have previously been measured from excised skin samples (Sanyal 1962, Suzuki et al 1971, Horakova and Beaven 1974). Biopsies prohibit the evaluation of tissue histamine concentration from the exactly same location during follow-up. The microdialysis technique enables repetitive collection of samples from the extracellular space of a certain area. The analysis of histamine from the eluate has been used successfully both in pigs (Rojdmark 2000) and in several studies in human (Anderson et al 1992, Horsmanheimo et al 1994, Church and Clough 1999, Saarinen et al 2000, Krogstad and Roupe 2001, Koppert et al

2001, Rukwied et al 2002, Petersen and Skov 2003). Histamine concentrations have shown to be independent of probe depth in skin (Andersson et al 1996). The microdialysis method collects about 35 % of the histamine in a standardized solution in vitro (Horsmanheimo et al 1994). Therefore, the true concentrations of histamine in tissue in this study are actually higher than the measured values.

The biggest disadvantage of using the microdialysis method is the incapability to collect very early samples. With the low flow rate of 3.3 µl/min and a collection time of 30 minutes a sufficient amount of eluate is collected in order determine histamine concentration twice from each sample. The average of these two samples was used in the analysis. Therefore the earliest possible sample to collect would be 30 minutes post burn regarding the microdialysis probes were placed prior to inflicting the burns. This was not done in this study in order to avoid damaging the probe with heat. On the other hand, this still would not enable collection of samples during the first minutes post injury, when the rapid rise in histamine concentrations in plasma has been seen (Suzuki et al 1971, Markley et al 1975, Yurt and Pruitt 1986).

Microdialysis proved to be an easy and reliable way to collect samples from burned sites. It is therefore applicable in further research both to determine other substances from a burned site and to monitor the changes induced by modifying treatment modalities.

6.4. Dielectric measurements in tissue water determination

It has been said almost 35 years ago that “there is no doubt that a method for quantitive measurement of edema would represent a significant contribution to our understanding of edema formation” (Heydinger et al 1971).

Edema formation is characteristic for burn injuries being one of the most typical findings locally in a small burn and generally in burns of greater magnitude correlating to the heat exposure time and thus to the depth of injury (Arturson and Jakobson 1985). Tissue water content is a significant parameter in the evaluation of local burn injury. However, edema only becomes visually apparent after a significant increase in tissue volume (Sowa et al 2001). Burn injury causes increased fluid flux from the vascular to the interstitial fluid compartment causing swelling (Lund et al 1988). Edema is maximal at 4 hours (Leape 1970, Heydinger et al 1971, Demling et al 1978) to 6 hours (Boykin et al 1980) post burn and starts to resolve by 24 hours, but resolves completely only after 6-7 days (Demling et al 1978). Edema in burned tissue increases the risk of infection due to lowering tissue pO_2 (Hunt et al 1974) and might cause progression of a partial thickness burn to a full thickness burn.

There is plenty of literature concerning thermal injury-induced edema. The earliest studies have concentrated on providing evidence of the increased capillary permeability (Netsky and Leiter 1943, Cope and Moore 1944, Sevitt 1958, Wilhelm and Mason 1960) or on trying to localize the site of vascular permeability (Wells and Miles 1963, Spector et al 1965, Ham and Hurley 1968, Cotran and Remensnyder 1968, Vegad and Lancaster 1973, Arturson 1979, Arturson and Jonsson 1979). Later the focus was on measuring the amount of edema formation (Leape 1968, Leape 1970, Heydinger et al 1971, Demling et al 1978, Arturson and Jakobson 1985, Lindahl et al 1993, Zdolsek et al 1998, Infanger et al 2004). Finally the emphasis was on the edema formation mechanisms on a cellular level or measuring interstitial and intracapillary pressures (Eriksson and Robson 1977, Zetterström and Arturson 1980, Lund and Reed 1986, Pitt et al 1987, Lund et al 1987, Lund et al 1989, Ferrara et al 1994, Kinsky et al 1998), or, on the other hand, on the different treatment modalities in order to minimize edema formation (Wiedeman and Brigham 1971, Little et al 1978, Sokawa et al 1981, Björk and Arturson 1983, Saria and Lundberg 1983, Demling et al 1984, Cetinkale et al 1997, Ferrara et al 1998, Kinsky et al 1998, Radke et al 2000, Barrow et al 2000, Inoue et al 2001, Räntfors and Cassuto 2003). In our study, the applicability of the dielectric measurements to quantify local edema in different depths of tissue after thermal trauma was assessed. Therefore, the focus in this part of the discussion will be on the edema measurements only.

Only one in vitro investigation with isolated skin samples has been published on the difference between the dielectric properties of normal and burned skin (Bhattacherjee et al 1995) where increased dielectric constant values were found in burned tissue. They concluded that the technique could be used for assessing the progress of skin condition after thermal burn injury.

There are some external factors accounting for the measurements with the open-ended coaxial probe (Burdette et al 1980). Since the skin is not truly flat, some air gap formation might occur between the probe and the skin if the contact is too soft, leading to too small dielectric values. If the pressure is too high, however, a change in tissue structure and water composition may occur. Due to water evaporation through the epidermis a long contact time increases the moisture content between the probe and the skin resulting in an increased dielectric constant. Therefore, a measurement time of 2 seconds per measurement was used. Also, in order to avoid differences in the technical performance of measurements by different individuals in this study, all measurements were performed by the same person.

In the present study local tissue edema was measured during a 72-hour period with the dielectric probes of 3 different sizes giving information from 3 different depths of tissue. The water content in the upper dermis in the superficial burns resembled closely that of unburned skin correlating to mild injury. However, it was increased at 8 hours post injury when the whole thickness of the skin was taken in account. The partial thickness burns demonstrated increased

tissue water in whole skin at 8, 24 and 72 hours post injury relating to a more severe permeability disorder. As the superficial and partial thickness burns do not involve the lower dermis, the reticular vascular plexus is intact being the site of elevated vascular permeability in skin. As suspected, the full thickness burns showed lower water content in the upper dermis throughout the follow-up period although the difference between the unburned control sites was significant only at 24 hours. With the damage extending down to subcutaneous fat the vasculature of the skin is destroyed leading to a smaller amount of tissue capable for fluid exchange (Sokawa et al 1981).

The time course of the development of edema has been considered to have a rather rapid onset with the maximum reached at 4-6 hours post injury and dissolving starting at 24 hours post injury. The earliest time points of the original measurements in our study were not included in the analysis due to the problem of multitesting. Although the water amount remained in a surprisingly steady level throughout the study the highest dielectric constant values were constantly measured at 24 or 48 hours post injury in skin and at 8 or 24 hours in fat.

One of the purposes of this study was to determine whether the dielectric measurements aided early differentiation of partial and full thickness burns. There are some earlier studies where burns of different depths are studied in relation to edema formation (Sevitt 1958, Cotran and Remensnyder 1968, Owen and Farrington 1976, Demling et al 1978, Sokawa et al 1981, Arturson and Jakobson 1985, Lund et al 1989, Sowa et al 2001, Shimizu et al 2002). In these studies the edema formation in superficial burns was compared to that of deeper burns. However, none of these studies compared the edema formation in different depths of skin. We found (with the superficial 5 mm probe) the tissue water content in superficial dermis in the full thickness burns significantly lower than in the 3 second burns during the first 24 hours and also lower than in the control site at 24 hours post injury. This relates to the early destruction of superficial dermis caused by a long contact time compared to mild injury with edema formation in the partial thickness burn. When the whole dermis was included in the measurements with the dermal 15 mm probe the full thickness burns had again lower tissue water content than the partial thickness burns at 8, 24 and 72 hours post injury.

Since visible edema forms in the subcutaneous tissues after all but the most trivial cutaneous burns, analysis of the subdermal tissues as well as the skin seems essential (Sokawa et al 1981). However, we demonstrated that marked subcutaneous edema develops identically in all depths of burns and therefore fat edema measurements can not be used in burn depth evaluation. The explanation of this might lie in the anatomy of skin vasculature. Since skin blood vessels originate from deeper tissues, the burn injury-related obstruction of the superficial vasculature may increase the intracapillary pressure in the remaining deeper vessels (Figure 17). This would lead to a bigger escape of fluid from the vessels to the interstitial space due to the permeability disorder. On the other hand, according to this theory, considering the blood flow to the area

remained the same, deeper burns would lead to an even higher intracapillary pressure in the remaining subcutaneous vessels compared to the more superficial ones resulting in more subcutaneous edema in these burns. This, however, was not seen in this study. Therefore, it is possible, that the maximum water accumulation in fat is accomplished early.

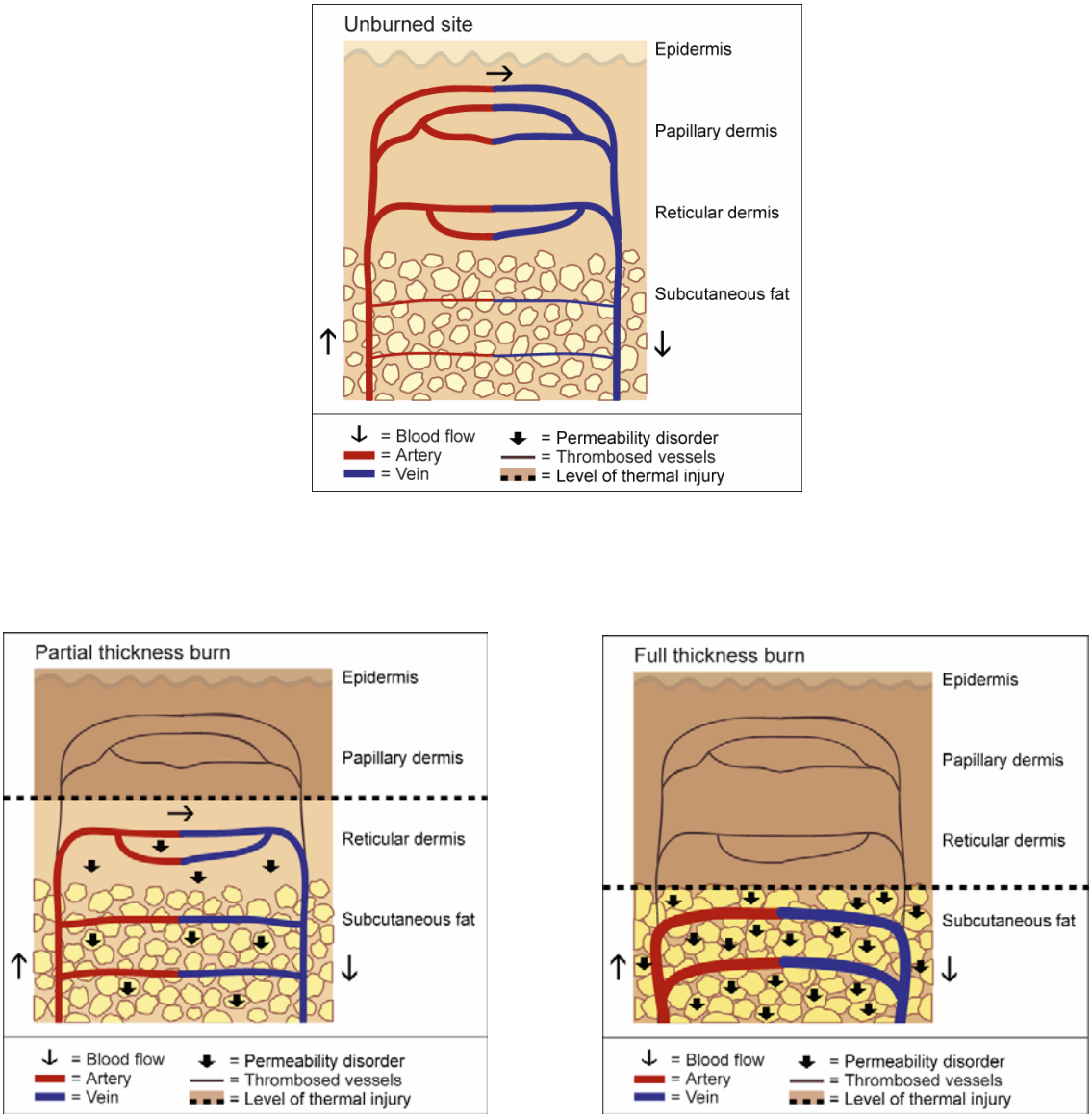

Figure 17. Schematic illustrations of a possible mechanism relating to the increased tissue water in subcutaneous fat after thermal injury. Unburned site, partial thickness and full thickness burns are presented individually (unpublished).

The threshold values for the dielectric measurements distinguishing the partial and full thickness burns at different times after injury was determined by performing the ROC curve analysis. These burn depths were chosen for the analysis because of their great clinical significance. In practise, deep burns need early excision and grafting when partial thickness burns mostly don't. A deep burn was classified as a positive finding as it is clinically important to be able to determine which patients benefit of early surgery. During the first 24 hours the superficial 5 mm probe had an excellent specificity (100 %) and PPV (100 %) and a good AUC (0.92). The AUC decreased slightly in time as did the specificity. On the other hand, the sensitivity increased towards the end of the study. The 15 mm dermal probe showed a specificity of 80-100 % throughout the study and good positive predictive values, too. The threshold values varied at different time points. Although these numbers can not be directly used in humans, these findings are of clinical importance. Early diagnosis of burn depth enables early treatment plans leading to either earlier discharge of patients or earlier surgical procedures thus shortening hospital stay.

The dielectric measurements provide us with a unique ability to quantify edema in different layers of tissue non-invasively in any anatomical location of skin.

6.5. The amount of labelled red cells and water in tissue

The site and time course of vascular injury has been widely studied with different methods. The purpose of this part of the study, however, was to determine the red cell and water amount in burned tissue samples and compare the results to the findings of the dielectric measurements and see whether they relate to the actual water amount and not hyperaemia at 24 hours after injury. The 24-hour study time is justified, because the microcirculation is compromized to the worst extent at 12-24 h (Arturson 1995).

The present study demonstrates that all burns induced a decrease in the number of labelled red cells (LRC) per 1 g of wet tissue in skin. Furthermore, the superficial and partial thickness burns caused a non-significant increase and the full thickness burns a significant decrease in the number of LRC's in subcutaneous fat compared to the non-burned control site at 24 hours post injury. The finding in full thickness injuries relates to the injury extending down to subcutaneous fat compromizing blood circulation. On the other hand, while the water content in skin increased only slightly, the water content in fat increased 64 %, 77 % and 67 % in the superficial, partial and full thickness burns, respectively, compared to the control site.

Several of the earlier studies on blood flow after thermal injury are of short follow-up time (Owen and Farrington 1976, Ferguson et al 1977, Hamar et al 1979, Taheri et al 1995, Mulligan et al 1994, Löfgren et al 1997, Jönsson et al 1998, Barrow et al 2000, Sowa et al 2001) with

follow-up times ranging from 30 minutes to 6 hours. The studies with a follow-up time of at least 24 hours have at that time point found decreased blood flow in full thickness burns in skin (Ehrlich 1984, Regas and Ehrlich 1992, Wang et al 1995, Sakurai et al 2002) and increased blood flow in subcutaneous fat at 72 hours post injury (Wang et al 1995, Sakurai et al 2002). Subdermal vascular dilatation has also been documented after a full thickness injury in rats (Ehrlich 1981).

The content of water in non-burned skin was 72.3 % and in subcutaneous fat 36.6 % which resembles that of human. The water content in skin increased only slightly but there was a significant increase in the water content in fat regardless of burn depth. This is supported by earlier studies (Sakurai et al 2002). The reasons contributing to subcutaneous edema are discussed above.

Finally, the clinical determination of burn depth is usually performed between 48-72 hours post injury. The earlier differentiation of partial and full thickness burns would allow earlier surgical interventions in full thickness burns and, on the other hand, earlier discharge of patients if surgery is not needed. These would lead to shortening of hospital stay and economical savings. Mistakes in burn depth determination lead to prolonged hospitalization for patients with deep burns and possible unnecessary operations for patients with superficial burns (Heimbach et al 1984). Also, in life-threatening burns, where donor sites are limited, it is crucial to distinguish between partial and full thickness burns. Our study showed that the dielectric measurements allow early differentiation of experimental partial and full thickness burns in pigs and are hence of clinical interest in human studies. The microdialysis method, on the other hand, enables sample collection from extracellular space in a way that has not been previously reported in burn literature. This method gives us an opportunity to more precisely study the local events in burn wounds, particularly the secretion or accumulation of mediators within the burn site, helping us to understand the complexity of a burn wound more clearly.

7. SUMMARY AND CONCLUSIONS

Based on the present study the following conclusions can be drawn:

1) A reproducible animal model to create superficial, partial thickness and full thickness cutaneous burns in pigs was obtained for experimental burn research purposes (I).

2) The microdialysis method is easily applicable in experimental burn research. The initial increase in tissue histamine concentration is directly correlated to burn depth. There is a secondary increase in tissue histamine concentration without an increase in histamine levels in plasma relating to a local, not a systemic event (II).

3) The dielectric measurements provide a sensitive method for examining tissue edema after thermal injury in different depths of tissue. A marked subcutaneous edema is found in all burns regardless of burn depth (III).

4) The dielectric measurements seem to enable early differentiation of partial and full thickness burns in this experimental burn model in pigs and are hence of clinical interest (III).

5) The increase in the dielectric constants after thermal injury relate to tissue water, not hyperaemia (IV).

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The progression of burn depth in experimental burns: a histological and methodological study

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Abstract

This study was designed to create a reproducible model for experimental burn wound research in pigs. Previously, the thicker paraspinal skin has been used. We used the more human-like ventral skin to create burns of different depths.

Contact burns were created to 11 pigs using a brass plate heated to 100 °C in boiling water. Different contact times were used to create burns of different depths. In pigs 1–6, the follow-up time was 72 h and in pigs 7–11 24 h. Burn depth was determined by histology. Histologically, samples were classified into five anatomical layers: epidermis, upper one-third of the dermis, middle third of the dermis, deepest third of the dermis and subcutaneous fat. The location of both thromboses and burn marks were evaluated, respectively.

The 1 s contact time lead to a superficial thermal injury, 3 s to a partial thickness and 9 s to a full thickness injury. A progression of burn depth was found until 48 h post-injury. The intra-observer correlation after repeated histological analyses of burn depths by the same histopathologist and the repeatability of burn depth creation yielded kappa coefficients 0.83 and 0.92, respectively.

Conclusion: a reproducible burn model for further research purposes was obtained.
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Keywords: Burn; Depth; Histology; Progression; Pig

1. Introduction

Treatment of a burn wound is dictated by its depth. In practice, the depth of the injury is evaluated by clinical judgement [1]. Superficial and very deep burns are not a challenge for burn depth evaluation. However, burns of indeterminate depth create challenges both in diagnostic matters and treatment modalities.

Histological wound biopsy would seem to be the most precise diagnostic tool for burn depth evaluation [1]. However, it has never become a useful tool for clinical practice, since it is expensive, time consuming and leaves a scar in the site of the biopsy [2]. Also, the dynamic changes in the burn site make both the timing of the biopsy and the evaluation of the microscopic changes troublesome [1].

The development of oedema and progressive vascular damage are characteristic for burn injuries [3]. Vascular patency has shown to be a reliable method for determining the depth of burn injury [4]. Accordingly, a progression of burn depth was found in deep dermal burns in humans while superficial burns remained stable during the 48 h follow-up.

As a part of a larger study to examine local events in experimental burns, we aimed to create a reproducible burn model of inducing burns of different depths. Previously, the thick dorsal skin of the pig has been used to create experimental burns [5,6]. The skin of pig and human is almost indistinguishable by physiological, anatomical and physical resemblance [7]. In the present study, the thinner, more human-like, skin of the ventral side of the pig was used.

2. Materials and methods

2.1. Anesthesia and monitoring

This study was approved by the Institutional Animal Care and Use Committee of the University of Kuopio. Three-month-old female Finnish landrace pigs ($N = 11$, 28–38 kg) were fasted for 48 h with free access to water

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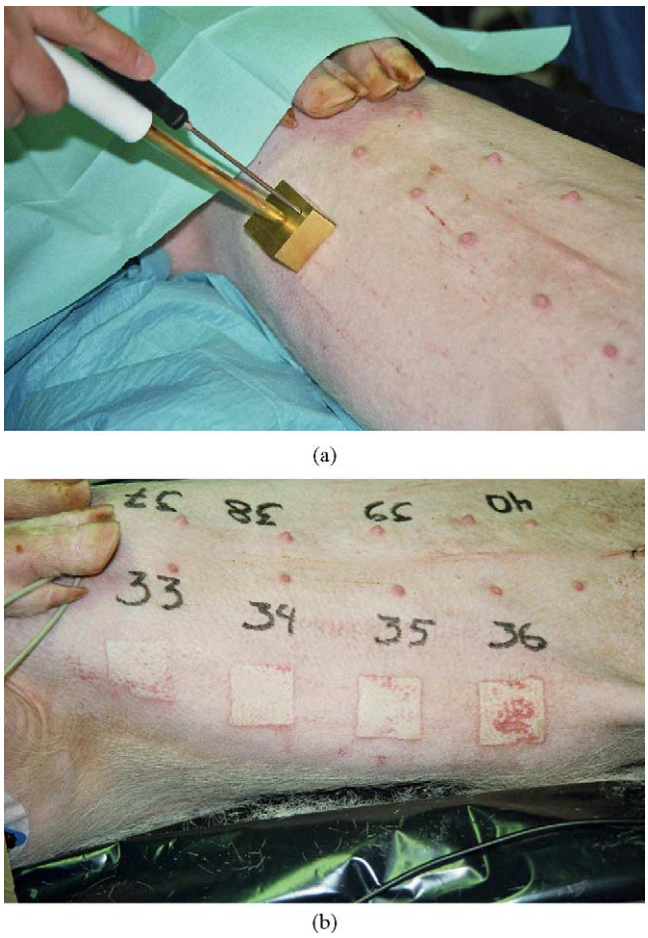
prior to the experiment. After premedication with atropine (0.05 mg/kg i.m.) and azaperone (8 mg/kg i.m) an ear vein was cannulated for administration of thiopenthal sodium (5–15 mg/kg i.v.) as induction of general anesthesia. Animals were intubated or tracheostomized and ventilated with a volume-controlled ventilator (Servo 900E, Siemens, Elema, Sweden) at a tidal volume of 10 ml/kg. Minute volume was adjusted to achieve normocapnia ($paCO_2$ 4.4–5.5 kPa, 34–41 mmHg). Fraction of oxygen in the inspiratory gas (FiO_2) was adjusted to keep arterial partial pressure of O_2 >13.3 kPa (100 mmHg). Positive end expiratory pressure of 5 cm H_2O was maintained throughout the study. Anesthesia was maintained with infusion of thiopentone (5 mg/kg/h). Pancurone (2–4 mg boluses i.v.) was administered for shivering when needed and fentanyl 30 μg/kg/h during the creation of burn wounds and 5 μg/kg/h thereafter for pain relief.

Right carotid artery and internal jugular vein were cannulated for blood pressure and central venous pressure (CVP) monitoring and blood sampling. Systemic and central venous pressures were recorded with quartz pressure transducers and displayed on a multimodular monitor and recorder (AS3, Datex-Ohmeda, Helsinki, Finland). Continuous information was collected automatically in two minute intervals (Clinisoft, Datex-Ohmeda, Espoo, Finland). All pressure transducers were zeroed to the level of the heart. Heart rate was continuously monitored with electrocardiogram. Haemodynamics were recorded at 15 min intervals. A urinary catheter was placed through a small incision in the lower part of the abdomen for urinary output measurements.

Animals received 50% glucose infusion which was adjusted to maintain normoglycaemia (5–7 mmol/l). Normovolaemia was maintained with Ringer's acetate (Ringersteril, Baxter, Vantaa, Finland) according to CVP 4–7 mmHg.



(a)

(b)

Fig. 1. (a) Creating a burn with a heated brass block. A digital thermometer is placed inside the block to measure the temperature of the block. (b) The 3 (marked 33), 6 (34), 9 (35) and 12 (36) second contact burn sites on right flank of the animal.

2.2. Experimental protocol

The ventral side of the body was shaved and washed with chlorhexidine solution (5 mg/ml). The burns were created by using a 4 cm × 4 cm brass block heated to 100 °C in boiling water (Fig. 1a). The temperature of the block was measured by having the tip of a digital thermometer (Lutron Thermometer, Taiwan TM-903) inserted in a hole of the block. By varying the contact time between the block and the animal skin, burns of different depths were created (Fig. 1b). Only the weight of the block was used to create the burns. No additional pressure was put on it. In order to avoid variations in creating the burns, one person (AP) created all burns. Two studies were performed.

Study 1: In pigs 1–5, four burns were created on each half of the ventral body 5 cm apart from each other using contact times 3, 6, 9 and 12 s. Contact times were modified from the previous investigation by Schomacker et al. [8] where thicker paraspinal pig skin was used. Thus, a total of eight burns were created on each animal, two of each contact time. Additional 10 burns (5 on each half of the ventral body) using a 1 s contact time were created on pig 6 to create more superficial burns and to test the repeatability of both the creation of superficial burns and the applied measurements. All burns were covered with moist saline dressings which were changed daily. There were two non-burned control sites in each animal, one in each side of the ventral torso. The follow-up time was 72 h. A 6 mm punch biopsy was taken at 2, 24, 48 and 72 h post-burn for histology, one from each quadrant of each burned site extending down to subcutaneous fat to evaluate the progression of burn depth. Thus, a total of four biopsies were taken from each burn site, one at each time point. The animals were kept sedated in the respirator throughout the study and were sacrificed with an i.v. overdose of magnesium sulphate.

Study 2: In pigs 7–11, three burns were similarly created on each side of the torso using 1, 3 and 9 s contact times. After creating the burns, the sites were covered with moist saline dressings. There was one control site in the middle of the ventral torso in each animal. The follow-up time was 24 h. The animals were kept sedated in the respirator throughout the study and sacrificed with an intracardial

injection of magnesium sulphate. A punch biopsy was taken after sacrifice from the center of the burned site for histological evaluation.

An experienced histopathologist (KK) analyzed the first 10 samples from Study 1 together with another pathologist, after which they agreed on the criteria of burn-related changes. Following this, all samples were analyzed by using standard hematoxylin and eosin-stained histological sections of 5 μm-thickness cuts from paraffin embedded samples by the same histopathologist. Evaluation of the samples was done blinded to the details of the sample. After the primary evaluation, a repeated analysis of 35 randomly selected samples was performed 18 months later by the same pathologist to test intra-observer reliability by calculating the kappa coefficient as described by Cohen [9].

Histologically, samples were classified into five anatomical layers: epidermis (level 1), upper one-third of the dermis (level 2), middle third of the dermis (level 3), deepest third of the dermis (level 4) and subcutaneous fat (level 5). The location of both thromboses and burn marks were evaluated respectively. Vascular patency was described as intact vessel walls with normal endothelial cells and no signs of cellular debris or tightly packed erythrocytes [4]. Epidermis was evaluated for burn artifacts (distortion of cell contour) and separation of epidermis from dermis (subepidermal blistering). Dermis was evaluated for histological separation or destruction of different cell layers of hair folliculi and vessel walls, microthrombi and neutrophils. Subcutaneous fat was evaluated for identical adnexal findings as in the dermis, thrombosis and fat necrosis. Burn depth at each evaluation was graded as 1–5 according to the depth of the deepest burn-related histological finding of each sample. In addition, skin thickness was measured from three different points of every sample from the dermo-adipous junction to the surface of the epidermis and presented as mean of the three measured values.

2.3. Statistics

Values are presented as mean (range). We used the Helmert contrasts, which compare the average of the response variable in a time point with the average of the following time point means in sequence. Also, the non-parametric Wilcoxon test was used for statistical analysis. The kappa coefficient [4] was calculated to test the intra-observer reliability in Study 1 and the repeatability of burn creation between Studies 1 and 2. All analyses were performed using SPSS for Windows (SPSS Inc., Chicago, IL, USA) program. A P-value <0.05 was considered statistically significant.

3. Results

The weights and burned areas of the animals are presented in Table 1. The pigs in Study 2 were heavier than in Study 1. All animals survived the experiment until sacrifice. No wound infections occurred at burned sites. Pig 2 created a clinically obvious pneumonia at post-burn day 2, after which prophylactic antibiotic treatment (cefuroxime 375 mg × 3 i.v.) was started to pigs 3–6.

Table 1
Demografics of animals in Studies 1 and 2

Pig	Weight (kg)	TBSA (cm^2)	TBSA (%)
Study 1			
1	30	128	1.9
2	26	128	2.1
3	30	128	1.9
4	31	128	1.8
5	33	128	1.8
6	33	160	2.2
Mean	30.5		1.9
Study 2			
7	38	96	1.2
8	40	96	1.2
9	36	96	1.2
10	34	96	1.3
11	36	96	1.2
Mean	36.8		1.2

TBSA: total burned surface area.

3.1. Haemodynamics

The data of haemodynamic measurements, temperature and blood gas analysis are presented in Tables 2 and 3. There were no time-related changes in the measured parameters.

3.2. Clinical inspection

By inspection, the 1 s burns appeared redder than the burns with longer contact times. In the 1 s burns, a very narrow, sharp-edged oedema area was clinically apparent at and around the burned area at 2 h whereas in 3–12 s burns oedema was apparent at 4 h being most obvious in the 9 s burns. At 12 h oedema was clinically diminishing in all burn sites and only minimal oedema was left at 24 h.

3.3. Histology

3.3.1. Study 1

The main histological changes are presented in Fig. 2a–c and the progression of burn depth in Fig. 3. There was no progression of burn depth evident in the 1 s burns. They were all graded as superficial dermal burns at all time points. According to the Helmert contrasts, the 3 s burns were significantly more superficial than the burns created with longer contact times ($P = 0.001$). Similarly the 6 s burns were significantly more superficial than the 9 and 12 s burns ($P = 0.006$). However, no difference was seen between the 9 and 12 s burns ($P = 0.104$). Also, there was a time-related

Table 2
Haemodynamic data and core temperature at different timepoints post-burn

Time (h)	CVPm		$ETCO_2$		HR		SAPm		Tc	
	mmHg	Range	kPa	Range	b/min	Range	mmHg	Range	°C	Range
2	6.0	4.4–7.0	4.1	3.8–4.1	76	57–129	76	55–86	36.8	36.6–37.1
4	5.9	4.0–7.5	4.2	3.8–4.4	75	52–100	77	72–86	37.3	36.1–38.2
8	6.8	5.6–8.0	4.1	4.0–4.5	56	48–69	76	71–79	38.2	37.4–38.6
12	6.3	5.2–8.0	4.1	4.0–4.5	57	54–66	76	67–80	38.0	37.9–38.8
24	6.0	4.6–7.0	4.1	4.0–4.3	61	64–100	79	64–100	38.3	38.2–38.6
36	5.7	4.2–6.7	4.2	4.0–4.5	62	58–72	87	80–90	38.4	38.0–38.7
48	5.3	3.1–7.5	4.1	3.8–4.3	66	59–78	86	67–100	38.4	38.1–38.7
72	6.2	3.0–7.5	3.9	3.3–4.1	76	75–100	73	71–93	38.3	38.2–38.5

Values are presented as mean and range. CVPm: mean central venous pressure; $ETCO_2$: end-tidal carbon dioxide; HR: heart rate; b/min: beats per minute; SAPm: mean systemic arterial pressure; Tc: core temperature.

Table 3
Data of the blood gas analysis at different timepoints post-burn

Time (h)	aB-BE		$aB\text{-}pCO_2$		aB-pH		$aB\text{-}pO_2$		$aB\text{-}sHCO_3^-$	
	mmol/l	Range	kPa	Range	kPa	Range	kPa	Range	mmol/l	Range
2	2.1	−5.3 to 4.6	4.8	4.7–5.1	7.5	7.3–7.5	23.7	22.1–26.3	25.2	19.4–27.9
4	4.4	0.9–7.9	4.4	4.2–5.1	7.5	7.5–7.6	22.6	21.0–24.5	26.9	23.7–31.7
8	3.3	1.6–5.8	4.3	4.1–5.0	7.5	7.5–7.5	22.7	21.0–24.4	25.6	24.1–28.9
12	2.4	0.1–4.4	4.6	4.3–4.8	7.5	7.5–7.5	22.7	20.3–23.9	25.0	23.4–27.2
24	1.6	−0.1 to 2.6	4.3	4.3–4.6	7.5	7.5–7.5	21.1	18.5–23.6	24.2	22.9–25.2
36	1.7	−0.8 to 2.4	4.7	4.4–5.0	7.4	7.4–7.5	22.5	16.9–24.4	24.6	23.8–25.1
48	2.2	−0.1 to 3.7	5.0	4.4–5.7	7.5	7.4–7.5	16.4	10.1–22.8	25.0	24.1–27.1
72	3.1	2.3–4.5	4.8	4.5–5.4	7.5	7.5–7.5	14.4	11.3–16.0	25.6	25.0–27.8

Values are presented as mean and range. aB-BE: arterial base excess; aB-Hb: haemoglobin; $aB\text{-}pCO_2$: arterial carbon dioxide tension; aB-pH: arterial pH; $aB\text{-}pO_2$: arterial oxygen tension; $aB\text{-}sHCO_3^-$: arterial bicarbonate concentration.

progression of burn depth during the study (Fig. 3). The depth of the injury was more superficial in all burn sites at 2 h post-burn compared to any later time point ($P < 0.0005$), and also at 24 h compared to later time points ($P = 0.015$), but there was no difference between 48 and 72 h post-burn ($P = 0.397$). Accordingly, progression of burn depth was histologically evident until 48 h post-burn.

Histologically, at 24 h post-injury, the 1 s contacts lead to a superficial dermal injury (level 2), the 3 s burn to a partial thickness injury (levels 3–4) and the 9 s burn to a full thickness (level 5) injury. Accordingly, these contact times and the follow-up time of 24 h were chosen for study 2.

3.3.2. Study 2

There were significant differences in burn depths between different contact times at 24 h post-injury. The 3 s burn was significantly deeper than the 1 s burn ($P = 0.004$) and the 9 s burn was deeper than both the 1 s burn ($P = 0.006$) and the 3 s burn ($P = 0.005$). The mean burn depths of different contact times are presented in Fig. 4.

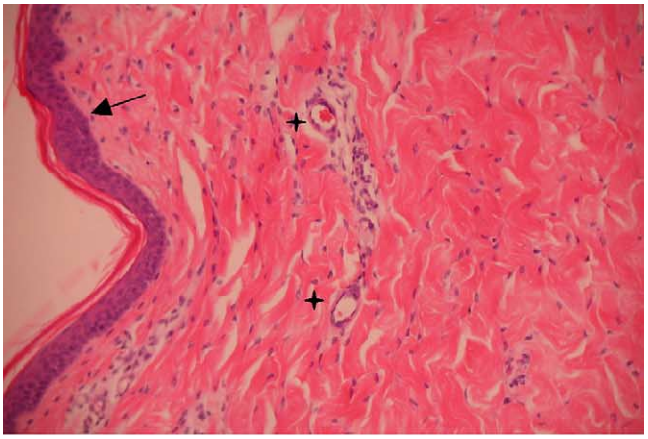
The kappa coefficient for intra-observer variation between two repeated analyses of 35 histological samples by the same pathologist in Study 1 was 0.83. The repeatability of burn depth creation between Studies 1 and 2 in the 1, 3 and 9 s burns yielded a kappa coefficient 0.92.

3.4. Skin thickness

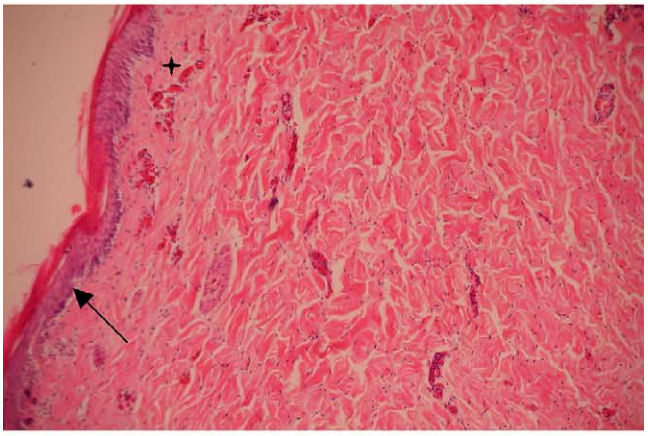
The changes in absolute skin thickness in Study 1 are presented in Fig. 5. The skin thickness of the non-burned control site remained unchanged (1.6 mm, range 1.5–1.73 mm) throughout the study. The skin thickness in the 1 s burns was greater compared to both the control site ($P = 0.005$ at all time points) and to the 3, 6, 9 and 12 s burn sites ($P = 0.005$–0.012) throughout the study period. The skin thickness in the 3, 6, 9 and 12 s burns was greater than the skin in the control site at all times ($P = 0.005$-0.037) expect the 3 s burn at 2 ($P = 0.285$) and 72 h ($P = 0.92$) and the 12 s at 72 h ($P = 0.767$). There were no differences between the 3, 6, 9 and 12 s sites.

4. Discussion

Histological evaluation has been considered the golden standard in burn depth determination despite the fact that it has many problems [1]. Burn wound is a dynamic entity consisting of progressive time-related changes. Therefore, timing of the evaluation is troublesome. The very early evaluation of the depth of irreversible injury may be difficult due to the ensuing progressive tissue damage [10]. Histology is



(a)



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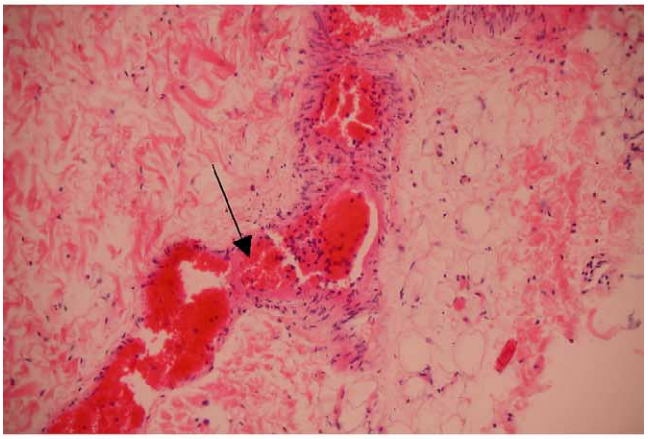

(c)

Fig. 2. (a) Normal pig skin from non-burned control area. Note the intact epidermal layer (arrow) and the patent vessels (asterix) (10×). (b) Subepidermal blistering (arrow) and hyperemia (asterix) in a 12 s burn 2 h post-burn (10×). (c) Thrombosis in the dermo-adipous junction. Note the mass (arrow) within the lumen of the vessel indicating old thrombosis. Also neutrophil infiltration present (20×).

also prone to errors due to the subjective nature of the assessment of different variables.

The main results in our study were that a reproducible model was obtained for creating experimental burns of different depths by using 1, 3 and 9 s contact times in pigs. It

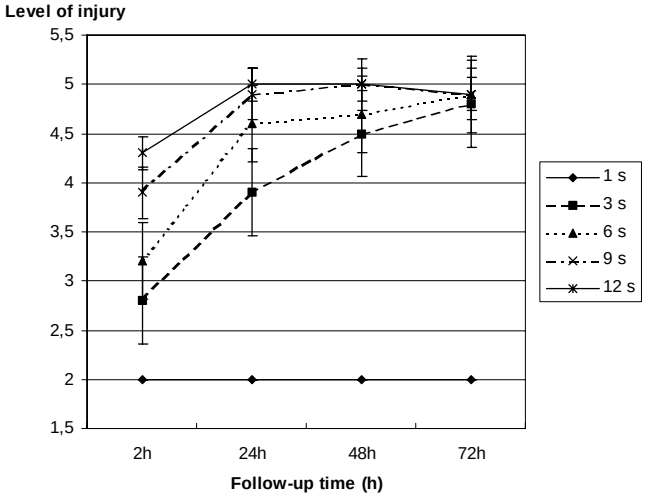

Fig. 3. Progression of histologic depth (±1 S.E.) of all burn sites during follow-up. Anatomic level of thermal injury is graded from 1 to 5.

was also found that there was no progressive tissue damage in the most superficial burns (1 s burns). In contrast, there was an increase in burn depth in the 9 and 12 s burns lasting up to 24 h post-burn and in 3 and 6 s burns lasting up to 48 h post-burn.

Several different animals have been used for burn research [5,6,10–17]. Pigs were used in the present study since pig skin has many similarities to human skin [5,7,17,18]. The absence of the intradermal muscular layer, panniculus carnosus, in pigs leads, as in humans, to closure of the wound by epithelial growth instead of contraction. Secondly, pig and human skin has the same relative thickness of epidermis and dermis, presence of epidermal ridges, distinct papillary dermis, similarities in both the vascularisation of the hair follicle and the structure of the collagenous tissue framework and a deep layer of subcutaneous fat [18]. In experimental burns, it is also important to choose an animal big enough to have flat surfaces to create uniform burns [17]. Previously, the thick dorsal paraspinal skin has been used [5,6]. We used the thinner ventral body skin as has been

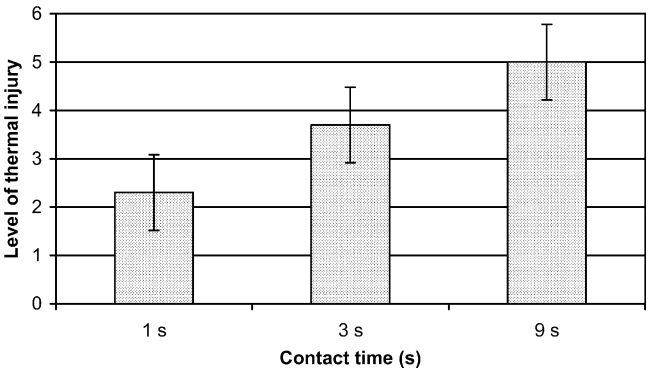

Fig. 4. Mean histological depth (±1 S.E.) of thermal injury in Study 2.

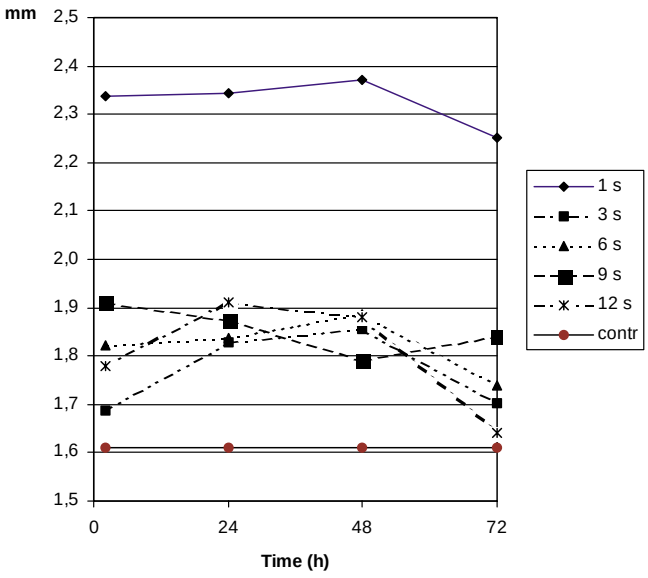

Fig. 5. Absolute skin thickness (mm) of different burn sites during follow-up.

used previously in small piglets [13]. The mean thickness of the ventral non-burned body skin was 1.6 mm, which is in accordance to previous studies, where skin thickness in 3–4-month-old pigs was 1.4 mm [19]. This is close to the thickness of human skin (1.44 ± 0.25 mm), measured from the thigh and forearm of 25–66-year-old people [20].

The mean surface area burned was 1.9% in Study 1 and 1.2% in Study 2. There was no burn-related need for fluid resuscitation and the animals were haemodynamically stable throughout the study (Table 2). Also, there were no changes in the thickness of the skin in the non-burned control sites indicating generalized oedema. Hence, it can be postulated that there were no significant systemic effects on the measured parameters caused by the thermal injury.

The importance of vascular patency in burn depth evaluation has been shown by Watts et al. [4], who found a good correlation between the histological evidence of vascular patency to clinical healing. Kahn et al. [21] suggested that it is important to evaluate blood vessels and their contents in determining the viability of the tissue. In Study 1, there were no thromboses in the 1 s burns and only in 15% of the samples in Study 2 within 24 h post-injury (10% in the superficial dermis and 5% in the middle dermis). When both vascular patency and other histological burn marks were evaluated, no progression of burn depth in these superficial burns was found. This is in accordance with the study of Watts et al. [4] where the depth of the superficial burns remained stable with time. However, in the 3–12 s burns we found a clear increase in depth with time, which has also been described before [4,12]. The final burn depth in the 9 and 12 s burns was obtained in 24 h post-injury when there was an increase in burn depth in the 3 and 6 s burns until 48 h. This might reflect the fact that in the 9 and 12 s burns the initial damage due to longer contact time already extends deep into tissue. In the 3 and 6 s burns, however, there must be a more clear ‘zone of stasis’ in the deeper part of dermis, where the ensuing vascular flow impairment leads to tissue ischemia, which might be devastating for already compromised cells.

The pattern of vascularisation of pig skin differs somewhat from human skin. It displays a lower, mid-dermal and subepidermal network, where the latter is less dense than in human [18]. Therefore, the direct interpretation of the results in human is limited. Due to the lack of pathologists experienced in evaluation thermal injuries histologically, an inter-observer reliability analysis could not be performed. However, the repeated analyses after 18 months by the same pathologist yielded an intra-observer correlation of 83%, which according to the definition of Landis and Koch [22] is considered almost perfect. Although all 1 s burns were created to the same animal and no comparisons could be made between different animals, the histological depth of all the 1 s burns were the same indicating a good repeatability of creating such burns. A good repeatability was also found in creating the 1, 3 and 9 s burns in Studies 1 and 2 yielding a kappa correlation of 92%, which is also considered almost perfect [22].

In conclusion, a reproducible model for creating superficial, partial thickness and full thickness thermal injuries in the skin of ventral body of a pig was obtained for further research purposes. This model is useful to examine more closely the local events and differences between burns of different depths in such an animal model where the skin closely resembles to human skin.

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II

Microdialysis for detection of dynamic changes in tissue histamine levels in experimental thermal injury

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Keywords: burn; histamine; microdialysis; edema; mediator

Summary

Histamine is an important mediator contributing to the edema formation after thermal injury. Tissue histamine concentrations have been previously determined by analyzing tissue biopsies. The microdialysis method enables continuous collection of samples from the extracellular tissue fluid. In this experimental burn study in pigs samples from the extracellular fluid for histamine analysis were collected from superficial, partial thickness and full thickness burn sites during a 24-hour period.

Results: There was a burn depth -related increase in histamine concentrations during the first 2 h post injury. Deep burns induced a more profound initial increase in tissue histamine concentration than the partial thickness and superficial burns. Histamine concentrations at all burn sites declined until 12 h post injury. There was a second rise in tissue histamine concentrations between 12 and 24 h post injury without a rise in plasma histamine concentrations. Histamine concentrations at all burn sites were higher than at the non-burned control sites.

Conclusion: The microdialysis technique is an easily applicable method of collecting on-line samples from burned tissue. This method provides a useful tool in investigating the effects of different treatment modalities on the secretion of substances into interstitial fluid within burned tissue.

Keywords: burn; histamine; microdialysis; edema; mediator

Introduction

Edema formation is characteristic to burn injury. Histamine is an important mediator contributing to the early phase of increased vascular permeability after burn trauma[1]. The secretion of histamine from mast cells leads to an enhancement of xanthine oxidase activity and increased production of free oxygen radicals, which damage microvascular endothelial cells leading to increased vascular permeability and edema[2]. In rats, plasma concentration of histamine immediately after burn is related directly to the extent of surface injury while the time-related profile is related to the depth of the injury[3]. In cats, a 30 % full thickness burn causes up to a three-fold increase in plasma histamine levels, with the peak concentration occurring within 30 min post injury[4]. Thermal injury increased the blood histamine levels but decreased the skin histamine levels at 5 min post burn in dogs[5]. The early rise in blood histamine was suggested to be primarily due to the release of histamine from the skin[5]. This is supported by the finding that severe burn injury initiates a higher increase in early post burn tissue fluid histamine levels than in moderate injury in rat paws[6]. The release of histamine closely parallels the development of edema and once edema is fully developed, no further release of histamine occurs[6]. A delayed (7-14 days post burn) rise in histamine content in rat and mouse skin after a superficial burn has also been described[7].

In practise, biopsies have been obtained in order to determine tissue histamine concentrations after thermal injury[5,6,7]. Recently, an alternative method for measurement of water soluble substances in the extracellular tissue fluid has been developed. With the microdialysis technique it is possible to continuously collect samples from the extracellular fluid[8]. The technique is based upon the principle that water soluble molecules of the extracellular space will passively move through the pores of the semipermeable membrane of the microdialysis probe. To our knowledge, this technique has not been previously described in burn wound research.

The aim of this study was to test the microdialysis technique in experimental burns by collecting extracellular fluid from burned tissue for analysis of locally secreted substances using histamine as the mediator analysed.

Materials and methods

Animal handling and anaesthesia

This study was approved by the Institutional Animal Care Committee of the University of Kuopio. Female Finnish landrace pigs (N=5, age 3 months, weight 28-38 kg) were fasted for 12 hours with free access to water prior to the experiments. After premedication with atropine (0.05 mg/kg i.m.) and azaperone (8 mg/kg i.m.) an ear vein was cannulated for administration of

thiopenthal sodium (5-15 mg/kg i.v.) as induction of general anaesthesia. Animals were tracheostomised and ventilated with a volume-controlled ventilator (Servo 900E, Siemens, Elema, Sweden) at a tidal volume of 10 ml/kg. Minute volume was adjusted to achieve normocapnia ($paCO_2$ 4.4-5.5 kPa, 34-41 mmHg). Fraction of oxygen in the inspiratory gas (FiO_2) was adjusted to keep arterial partial pressure of O_2 >13.3 kPa (100 mmHg). Positive end expiratory pressure of 5 cmH_2O was maintained throughout the study. Anaesthesia was maintained with infusion of thiopentone (5 mg/kg/h). Fentanyl was administered 30 μg/kg/h during the creation of burn wounds and 5 μg/kg/h thereafter. Pancurone was admistered in 2-4 mg boluses i.v. when needed for shivering. Body temperature was monitored via the Swan-Ganz catheder. The animals were kept sedated on the respirator throughout the study after which they were sacrificed with an intracardial injection of magnesium sulphate solution.

Hemodynamic monitoring

Right carotid artery and internal jugular vein were cannulated for blood pressure and central venous pressure (CVP) monitoring and blood sampling. Systemic arterial and central venous pressures were recorded with quartz pressure transducers and displayed on a multimodular monitor and recorder (AS3, Datex-Ohmeda, Helsinki, Finland). The data was registered automatically in two minute intervals (Clinisoft, Datex-Ohmeda, Helsinki, Finland). All pressure transducers were zeroed to the level of the heart. Heart rate was continuously monitored with electrocardiogram. Hemodynamics was recorded at 15 min intervals. A urinary catheter was placed through a small incision in the lower part of the abdomen for urinary output measurements. Animals received 50 % glucose infusion which was adjusted to maintain normoglycemia (5-7 mmol/L). Normovolemia was maintained with Ringer's acetate (Ringersteril, Baxter, Vantaa, Finland) according to CVP 4-7 mmHg.

Experimental protocol

The ventral side of the body of the pig was shaved and washed with chlorhexidine solution (5 mg/ml). The burns were inflicted by using a 40 x 40 x 25 mm brass block, weight 530 g, heated to 100 ºC in boiling water as described earlier[9]. Contact times of 1, 3 and 9 seconds were used between the block and the animal skin to create superficial, partial thickness and full thickness burns, respectively[9]. A total of six burns were created per animal, one of each depth on both sides of the ventral body. There was a non-burned control site in the middle of the body (Figure 1). Burns were covered with 0.9 % saline dressings to prevent them from drying.

Microdialysis and histamine assay

After creating the burns, microdialysis was performed by using a CMA/Microdialysis Apparatus (Stockholm, Sweden) with a CMA100 probe (shaft 25 mm, membrane length 10 mm, a diameter of 0.5 mm, molecular weight cut-off limit of 20 kDa) as in histamine studies in humans[10]. A total of three probes can be connected to each microdialysis pump. A thick needle (Intraflon 2, 16G, ∅ 1.6 mm, Vycon, Ecquen, France) was inserted from one corner of the burned area towards the opposite corner, where the tip of the needle was pushed through the skin. The needle was removed and probe was inserted inside the lumen of the cannula, which was then pulled out from the insertion site leaving the tip of the probe in the middle of the burned area at the dermis-subcutis interface. Three CMA/Microdialysis Apparatuses and 7 probes were used simultaneously for each burned animal: 1 probe was inserted into each burned area (6/animal) and 1 probe identically to the non-burned control site (Figure 1). The positioning of each probe takes about 1 minute. The flow rate was set at 3.3 μL/min which gives a relative recovery of about 35 % for histamine in vitro[11]. Eluate fluid for histamine analyses were collected from burned animals at 1, 2, 6, 12 and 24 h post injury using a collection time of 30 minutes for each sample. Hence, the first sample collection started at 30 minutes post burn. Samples for the determination of plasma histamine concentrations were collected from a venous cannula before creating the burn and at the same time as the microdialysis samples. The microdialysis probe was not inserted prior to initiating the burn trauma due to the risk of damaging the probe with heat. Histamine assay was performed by the radio enzyme method using tritium-labelled S-adenosylmethione (15 Ci/mmol, Amersham International, Amersham, UK) and liquid scintillation counting for measurement of the radioactivity[12]. At the end of the experiment all the burned sites were excised with the microdialysis probe still in place. In order to verify the correct location of the probe, the skin and subcutaneous fat were separated sharply with scissors.

Statistics

The histamine concentrations are presented as median $\pm$ quartiles. The correlation co-efficient (R) was calculated with the Excel for Windows programme. A non-parametric Wilcoxon test was used for statistical analysis. All analyses were performed using SPSS for Windows (SPSS Inc, Chicago, IL). A P-value <0.05 was considered statistically significant.

Results

All animals survived the experimental period. Hemodynamics and temperature remained stable during the study (data not presented). There were no signs of wound infections or technical problems concerning the sample collection. The mean surface area burned per animal was 1.2 %

of the total surface area of the animal. The mean temperature of the brass plate after creating the 1, 3 and 9 sec burns were 96.2, 94.5 and 91.6 °C, respectively. When dissecting the burned areas, every microdialysis probe was located in the dermis-subcutis interface.

Microdialysis

The histamine concentrations in the microdialysis eluate and in plasma are presented in Figure 2. There was a good correlation between the contact time and the tissue histamine concentration at 1 and 2 h post burn with correlation coefficients (R) of 0.999 and 0.927, respectively (Fig.3). At one hour post injury the histamine levels in the eluate in the 9 sec (9S) burns were significantly higher than in the 1 second (1S) and 3 second (3S) burn sites ($p=0.003$ and 0.019, respectively). At 2 hours the 9S site had higher histamine levels than other burn sites (9S vs 1S: $p = 0.01$, 9S vs 3S: $p = 0.003$). At 6 hours the histamine concentration of the 9S site was higher compared to the 1S and 3S sites ($p = 0.03$ and 0.002, respectively). At 12 and 24 hours there were no differences between any burn sites. At all time points all burn sites had higher histamine concentrations than the control sites ($p = 0.012$-0.034) except the 3S at 6 hours. There was a significant decrease in histamine concentrations between 2 and 6 hours post burn in all burn sites (1S and 3S, $p = 0.007$; 9S, $p = 0.008$). No changes were found between 6 and 12 hours. However, between 12 and 24 hours post injury there was a significant increase in tissue histamine concentrations in all burn sites (1S and 3S, $p = 0.007$; 9S, $p = 0.028$). The highest concentration of histamine in the end of the study was found in the 1S and 3S sites and the lowest concentration in the 9S, although the differences between different burn sites at this time were not statistically significant.

Plasma histamine concentrations

Thermal injury caused an increase in histamine concentration until 2 hours post injury (Figure 2). Thereafter, the histamine concentrations decreased reaching a plateau at 12 hours post injury. There was no late increase in serum histamine level as was seen in the burn sites.

Discussion

Histamine has been considered an important local mediator in burn related edema formation[1]. The suggested pathways to increased local permeability are through nitric oxide release and/or stimulation of oxygen free radical formation[13]. Previously, tissue histamine concentrations have been mainly determined by analysing tissue biopsies[5,6,7]. With the microdialysis method it is possible to collect fluid dialysed from the extracellular fluid without biopsies. This method has been documented in other fields of research both in pig[14] and human skin[8,10,11,15-19]. It has also

been shown to be a useful tool for investigating the activation of mast cells and systemic release of histamine by muscle relaxants in humans[17].

The main finding of this study was that the microdialysis technique is applicable in burn wound research. We were able to detect burn depth-related changes in tissue histamine levels. Thermal injury resulted in high initial tissue histamine concentrations which decreased until 6 hours post burn. At one hour post burn the histamine concentrations in tissue were directly related to the heat exposure time (Fig.2). A second rise in tissue histamine levels was inversely related to the burn depth without a rise either in the control site or in plasma.

Mast cells are widely distributed in the connective tissue of skin and in mucous membranes of the digestive and respiratory tract, especially near small blood vessels[20]. In normal skin, mast cells occur in the greatest density in the superficial dermis[15]. Thermal trauma causes degranulation of mast cells and histamine liberation[20,22]. In our study, the highest concentration of histamine in the microdialysis eluate was seen in the one hour samples post injury. At that time the histamine levels in the full thickness (9S) burns were significantly higher than in the non-burned control site and the superficial (1S) burns (Figure 2), which is in accordance with previous studies where tissue histamine levels increased more in severe thermal injury compared to moderate ones[6]. The tissue histamine levels were elevated also in the non-burned control site indicating either a systemic effect caused by the burns or a reaction caused by the insertion of the microdialysis probe. Previously, an up to 47 % rise in non-burned skin histamine levels has been reported after a 20 % full thickness burn in rats[23].

The early rise in plasma histamine in cutaneous burns has been considered to be primarily due to the release of histamine from the skin[4,5]. Also, interstitial histamine in the dermis has been found to represent specifically the local tissue concentration[16]. In the present study, the highest concentration of plasma histamine was seen later than the highest concentration in tissue**.** This supports the previous suggestion of the early rise in blood histamine being primarily due to the release of histamine from the skin[5]**.** Deep burns induce a higher initial tissue concentration of histamine than superficial burns[6]. It is likely that deep burns destroy more mast cells than superficial ones leading thus to an enhanced liberation of histamine. However, this seems to be in conflict with the fact that the vast majority of skin mast cells are located in the upper dermis[15]. Accordingly, if the upper dermis of the burned site would be the only source of histamine in skin, a full thickness burn would yield the same tissue histamine concentration than a burn that is limited to the superficial third of the dermis. Therefore it is possible, that histamine is released from the middle and deep dermis also. Another explanation is a more profound permeability disorder of deep burns allowing enhanced histamine leakage from the damaged blood vessels. Hence, the origin of the high tissue concentration of histamine in deep burns could be partly due to the histamine from plasma instead of local mast cells since the blood vessels in this depth of tissue are not totally obliterated at 24 hours post burn due to the progression of burn depth until

48 hours[9]. Basophils in blood can also a source of histamine. In addition, as the depth of the burn injury is known to progress in time[9,24], the fact that no further increase in tissue or plasma histamine levels are found during the first 12 hours does not support the role of dermal mast cells as the sole source of histamine release in deep burns.

There was a late increase in tissue histamine concentrations in all burn sites beginning after 12 hours post injury. The median histamine levels were 20, 19 and 8 times greater at 24 hours in the superficial, partial and full thickness burns, respectively, compared to the control site (Fig. 2). However, the differences between burn sites were not statistically significant. The fact that there was no simultaneous increase in plasma histamine concentrations, suggests a local event rather than a systemic one responsible for the late increase in tissue histamine concentrations. The delayed increase in tissue histamine levels might be due to the local progressive inflammatory response, which is known to occur in burns[25]**.** It is also possible, that the late histamine secretion from the originally surviving mast cells is triggered by other mediators, like anaphylatoxins[26]. Partially damaged mast cells liberate histamine, but are able to survive and make new histamine granules. Furthermore, when the initial vasoconstriction subsides[24], the increasing blood flow might bring other mediators to the wound site triggering the release of histamine locally. The possible release of neuropeptides, like substance P, from the damaged nerve endings, may also be involved in delayed histamine deliberation[27]. This neuropeptide induces vasodilatation and vascular permeability by stimulating endothelial cells to round up, vascular smooth cells to relax, and mast cells to release histamine. Also, the subsiding edema at burn sites might lead to increased histamine concentrations in tissue. A late increase in tissue histamine levels has been reported earlier at 2 days[22] in rats and at 14-21 days[7] in mice post injury. The follow-up times of different histamine studies are inconsistent varying from 120 minutes to 21 days[2-7,23,28]. In a similar follow-up time with the present investigation with rat paws, no late increase in tissue histamine levels after thermal injury was noted[6].

Plasma histamine concentrations increase within 1 minute after thermal injury in rats[4]. In the present study, plasma concentrations were elevated already in the pre burn samples. This is likely due to the initiation of general anaesthesia and cannulation of the animal before creating the burns[4]. Hence, the present pre burn plasma histamine concentration does not give the true baseline level of plasma histamine concentration in these animals. The highest concentration was measured at 2 hours, which is in accordance with the peak concentration of plasma histamine in rats 2 hours post injury after a 30 % partial thickness burn[4]. However, earlier peak concentrations have also been reported[2,3,5,6,28]. We found no late increase in plasma histamine levels, which may be due to stable general anaesthesia during the study period and no further manipulation of the animal or the wound.

With the microdialysis method we were unable to collect very early samples due to both the collection time of 30 min of the samples and the possibility to insert the probe only post injury. With the microdialysis technique the collection time of samples is usually 15 to 120 minutes, depending both on the volume of eluate needed for the laboratory analysis and the flow rate of the perfusion fluid limiting the collection of very early samples. Due to the risk of destroying the microdialysis probe due to excessive heat when contact burns are created the probes were inserted post burn. Hence, no pre burn tissue histamine concentrations were measured.

As the microdialysis method has been shown to collect about 35 % of the histamine in a standardised solution in vitro[11], it can be postulated that the concentrations of histamine in tissue in this study are actually higher than the measured values hence being closer to those in plasma. Furthermore, the histamine liberation caused by the insertion of the microdialysis probe has been found to last up to about 40 minutes[29]. This, in addition to the manipulation of the animal prior to creating the burns, explains at least partly the increase in histamine values in the eluate from the non-burned control sites in the beginning of the study. Since burns of three different depths were created to each animal, the plasma histamine levels in superficial, partial thickness of full thickness burns separately could not be determined. Also, because tissue histamine concentrations were not additionally analysed from tissue biopsies, blister fluid or local lymph, the histamine concentrations from the dialysate in this study could not be compared to concentrations analysed by other methods.

To our knowledge, this is the first time the microdialysis technique has been reported in burn wound research. It is an easily applicable method of collecting on-line samples from burned tissue. This method provides a useful tool in investigating the effects of different treatment modalities on the secretion of substances into interstitial fluid within burned tissue.

References:

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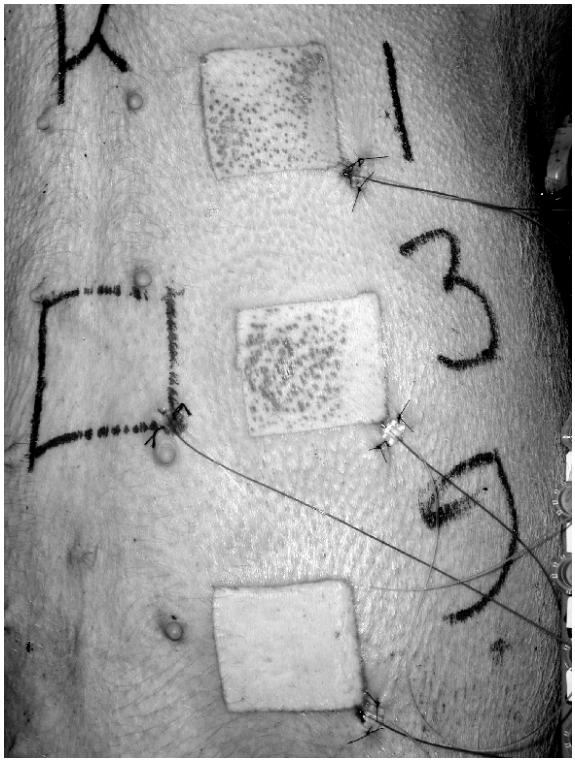

Figure 1. The three different burn sites (1, 3 and 9 second contact burns) and the non-burned control site in the ventral body of the pig. Each area has the microdialysis probe (blue) inserted in the dermis-subcutis interface from the corner of each area.

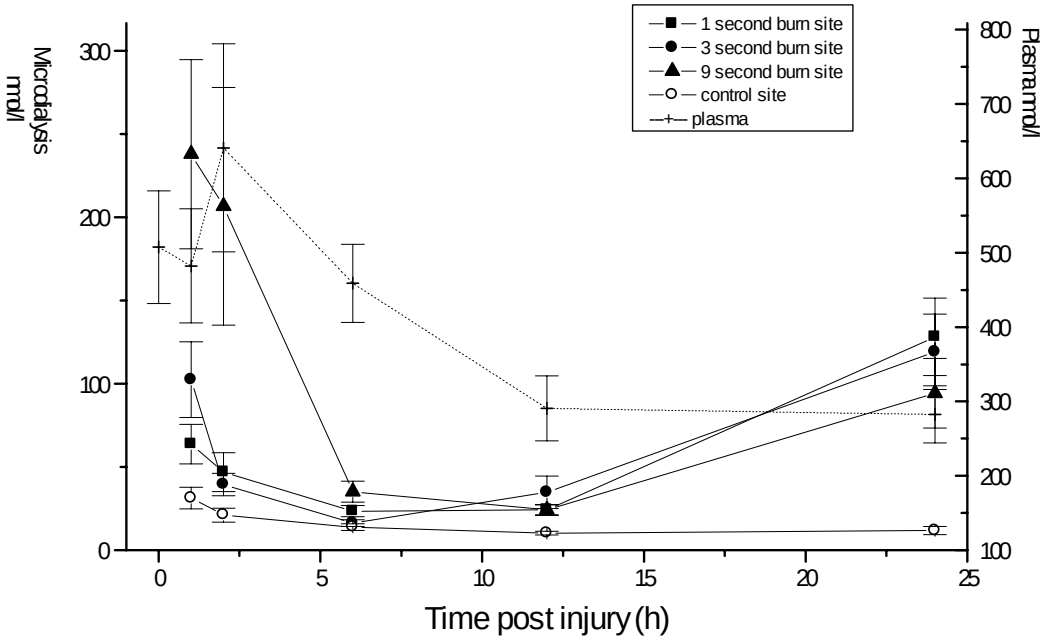

Figure 2. Histamine levels (median ± quartiles) in the microdialysis eluate and plasma at different time points.

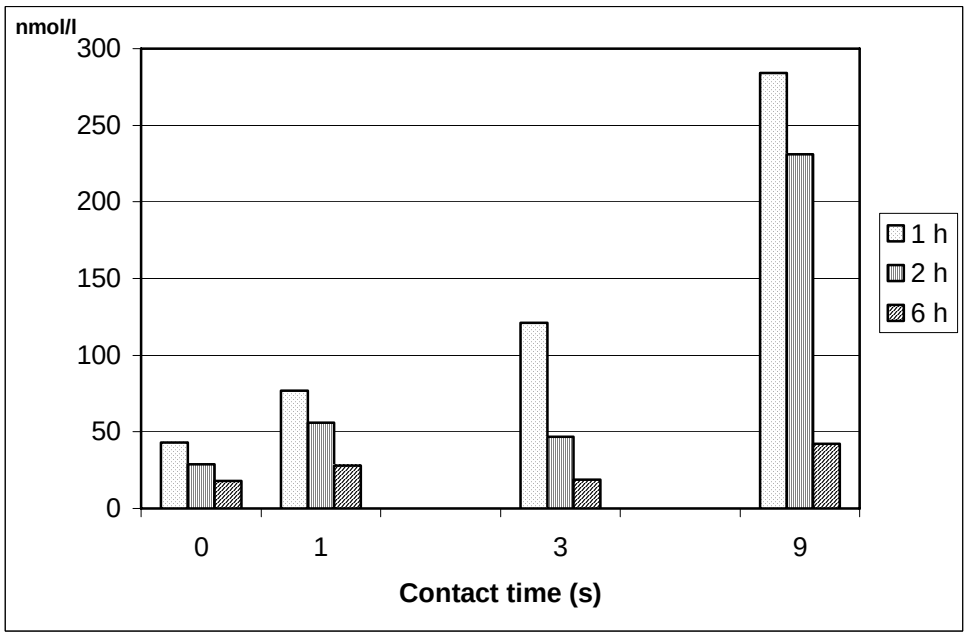

Figure 3. Burn duration -related histamine concentration in tissue presented as a function of burn duration at 1, 2 and 6 h post injury. The values at 0 seconds represent the non-burned control site

III

Dielectric measurement in experimental burns:

A new tool for burn depth determination?

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Abstract

Background: There has been a lack of methods that provide quantitive information of local tissue edema after burn injury. The non-invasive dielectric measurements provide this information. The measured value, the dielectric constant, is directly related to the amount of water in tissue. With probes of different sizes the measurements give information from different depths of tissue. The aim of this study was to characterize edema formation at different depths of tissue and examine whether the dielectric measurements could be used to distinguish partial and full thickness burns in pigs.

Methods: Experimental animal study with pigs (N=6), where dielectric measurements were performed to superficial, partial and full thickness burns during 72 hours.

Results: There was an increase in tissue water content in the superficial dermis in the partial thickness burns at 48 hours. In whole dermis the superficial burns resulted in increased tissue water content at 8 hours and the partial thickness burns at 8, 24 and 72 hours. In deep burns, the water content was significantly decreased in the superficial dermis at 24 hours. All burns resulted in a considerable increase in fat water content. The dielectric probes could be used to differentiate partial and full thickness burns as early as 8 hours post burn. The ROC analysis indicated 70-90 % sensitivity and 80-100 % specificity after 8 h for the measurements.

Conclusion: The dielectric measurements provide a sensitive and non-invasive method for examining tissue edema and differentiating partial and full thickness burns in experimental burns and are thus of clinical interest for early burn depth determination.

Introduction

Edema formation is characteristic to burn trauma. Burn injury causes increased fluid flux from the vascular to the interstitial fluid compartment causing swelling (1). Post burn edema formation correlates to the heat exposure time and thus to the depth of injury (2). Edema is maximal at 6 hours post burn and starts to resolve by 24 hours, but resolves completely only after 6-7 days (3). Edema in burned tissue increases the risk of infection due to lowering tissue pO_2 (4) and might cause progression of a partial thickness burn to a full thickness burn (2).

The dielectric parameter, the so-called dielectric constant of a biological material at a high radiofrequency, is useful in the assessment of skin and subcutaneous water content (6,7). Accordingly, high-frequency electromagnetic fields are guided into skin where they react with the water molecules in tissue. The dielectric constant is calculated from the reflected wave and it increases linearly with the increase of tissue water content in high water-containing tissues like skin. The dielectric constant of air is 1.0 and water 78.5 (7). Using an open-ended coaxial probe (Fig. 1), the effective depth of measurement is dependent on the dimensions of the dielectric probe (6). Measuring the dielectric constant of burned skin with probes of different sizes might offer specific information of the edema in the skin and subcutaneous tissues and its possible progression to deeper tissues. Only one in vitro investigation with isolated skin samples has been published on the difference between dielectric properties of normal and burned skin (8).

In order to evaluate the dielectric measurements with the open-ended coaxial probe technique at high radiofrequencies in the assessment of the extent of burn injury, an established skin burn model using pigs (9) was adopted. Clinical evaluation of burn depth is relative and unreliable. In clinical practise the aim of burn treatment is for early burn depth determination leading to early excision and grafting when indicated. The aim of this study was firstly to investigate the edema formation at different depths of tissue induced by superficial, partial thickness and deep burns and secondly whether the dielectric constant can be used in early burn depth differentiation between partial and full thickness burns.

Materials and methods

The study was approved by the Institutional animal care and use committee of the University of Kuopio, Finland. Six female Finnish landrace pigs (28-38 kg) were premedicated, cannulated, anaesthetized and monitored as described previously (9). The animals were kept on the respirator in order to standardize the general conditions as thoroughly as possible. Superficial, partial thickness and full thickness contact burns were created with a brass plate heated up to 100° C on both sides of the ventral body of each pig by using 1, 3 and 9 second contact times, respectively (9). Each animal had two non-burned control sites (CS), one in each side of the ventral body.

At 8, 24, 48 and 72 hours post burn non-invasive dielectric measurements were performed from the centre of each burn and control site by placing 3 probes (Delfin Technologies Ltd, Kuopio, Finland) of different sizes successively on the injured skin. No pressure was applied, only the weight of the probe was used. The diameters of the probes were 5, 15 and 30 mm. The effective measurement depths of these are 0.5, 2.5 and 5.0 mm yielding information from the upper dermis, whole dermis and dermis-subcutaneous fat, respectively (6). A high-frequency electromagnetic field of 300 MHz was transmitted through a coaxial probe onto the surface of the skin. The reflected electromagnetic wave was detected by the probe (Fig 1.) and analysed by the HP8753C network analyser (Hewlett Packard Co, USA). Each measurement lasts for 2-3 seconds and is painless. The electrical quantity, the so-called dielectric constant calculated from the reflected wave, is directly related to tissue water content (7). Therefore, changes in the dielectric constant represent changes in tissue water content. The animals were kept sedated in the respirator throughout the study and were sacrificed at 72 hours post burn with an overdose of intravenous magnesium sulphate.

Statistics

The results are presented as mean ($\pm$ 1SD). The non-parametric Wilcoxon signed rank test (2-tailed) was used for statistical analysis using the SPSS for Windows programme. A P-value < 0.05 was considered statistically significant after a Bonferroni correction. The receiver operating characteristic (ROC) curve analysis with the corresponding area under the curve (AUC) was performed and positive and negative predictive values (PPV, NPV) calculated with the MedCalc Version 7.4.1.0 for Windows programme. In this study the ROC analysis was used to determine the cutting point for deciding whether the burn is partial thickness or full thickness burn. The AUC value varies between 0.5 and 1.0 where the area being as close to 1.0 is desirable. The PPV defines the probability of a positive finding to be truly positive and NPV identically the credibility of a negative finding.

Results

All animals survived the experiment until sacrifice. No infections occurred at burned sites. Pig No 2 created a clinically obvious pneumonia at post burn day 2, after which prophylactic antibiotics (cefuroxime 375 mg x 3 i.v.) were started to the rest of the animals.

Edema formation

In the superficial (1S) burn site, there was no difference in the water content in the upper dermis (Fig. 2a) compared to the control site. In the whole dermis (Fig. 2b), however, the water content was increased at 8 h compared to the control site. There was a significant increase in tissue water content when the subcutaneous fat was also included in the measurements (Fig. 2c). The partial thickness (3S) burns (Fig. 3a-c) resulted in rather similar findings as the superficial burns with the exception of increased tissue water in whole dermis at 24 and 72 hours, also. On the other hand, the full thickness burns showed lower water content in the upper dermis (Fig. 4a) throughout the follow-up period although the reduction was significant only at 24 hours. There were no differences compared to the control site in whole dermis (Fig. 4b), but the tissue water content was very high again in the subcutaneous fat (Fig. 4c).

Differentiation of partial (3S) and full thickness (9S) burns

Superficial (5 mm) probe

The tissue water content in superficial dermis in the 9 second burn sites was significantly lower ($p = 0.028 – 0.048$) than in the 3 second burn sites during the first 24 hours (Fig. 5a) and lower than in the control site ($p = 0.028$) at 24 hours post injury (Fig. 4a).

Dermal (15 mm) probe

The 9S burns had lower tissue water content in whole dermis (Fig. 5b) than the 3S burns at 8, 24 and 72 hours post injury ($p = 0.028 – 0.048$).

Deep (30 mm) probe

The tissue water content at all burn sites in subcutaneous fat (Fig. 2c, 3c and 4c) were significantly higher than at the control site ($p = 0.02$-0.036) except in the 1S burn site at 72 hours post injury. There were no differences between burn sites.

ROC analysis, AUC, PPV and NPV

The ROC analysis was performed in order to find the threshold values for the dielectric measurements distinguishing the 3S and the 9S burns at different times after injury. The results of the ROC analysis are presented in Table 1. A deep burn was classified as a positive finding. At 8

hours the superficial 5 mm probe had an excellent specificity (100 %) and PPV (100 %) and a good AUC (0.92). The AUC decreased slightly in time as did the specificity. On the other hand, the ability of the superficial dielectric measurements to find true positive findings (=sensitivity) increased towards the end of the study. The positive predictive values increased between 24-72 hours with the 15 mm probe. The negative predictive values (NPV) altogether were not as good as PPVs.

Discussion

Local measurements of edema in tissue after thermal injury have been troublesome. Several different methods have been described (2,3,11,16) but none of them has reached clinical value in determining burn depth. The present dielectric measurements using high-frequency electromagnetic fields result in a numeric value, the dielectric constant, which is directly related to the amount of total tissue water at a precise area.

The main findings of this study were that 1) the dielectric constant, e.g. water content, in burns of different depths varies; 2) all burns result in a high water content in the subcutaneous fat; 3) it is possible to distinguish burns of different depths from each other and from the non-burned control site with this method and 4) the partial and full thickness burns can be distinguished from each other already after 8 h post injury with high sensitivity and specificity indicating the possible clinical value of these measurements.

Several methods have been used to measure edema after thermal injury (3, 10-18). The water-specific dielectric method has a unique quality of enabling assessment of tissue water content non-invasively from different depths of tissue (6). One measurement lasts only a few seconds and can be done bedside with a small compatible device (7). The dielectric constant, calculated from the electromagnetic wave reflected from tissue, increases with the increase of tissue water content giving information of the local amount of edema in tissue.

The 1 and 3 second burns resulted in rather similar water distribution in tissue water in all depths of tissue. The partial thickness burn demonstrated a more obvious increase in the water content in whole dermis compared to the superficial burn (Fig. 2b and 3b). This might relate to the fact that the level of injury in partial thickness burns progresses until 48 hours post injury while there is no actual progress in burn depth in superficial injuries (9). On the other hand, the full thickness burn showed marked dryness in the upper dermis correlating to necrosis of this tissue layer (Fig. 4a).

In practise, evaluation of the depth of the injury is often done by naked eye during post burn days 2-3. However, this is not an objective way of determining burn depth. In addition to histological evaluation (9,19,20), several different methods have been described determining the depth of

thermally induced tissue damage (21-27). Previously, the use of the dielectric method has been described in experimental burn trauma literature only in isolated skin samples at frequencies 1-100 MHz (8). When performed with 300 MHz, it specifically gives information about the total amount of water in tissue consisting of both bound and free water (28). As post burn edema formation correlates to the heat exposure time and thus to the depth of injury (2) it is of great importance to be able to measure the amount of water in different layers of tissue.

Previously, the thickness of the non-burned ventral body skin of farm swine has been measured to be 1.6 mm and up to 2.4 mm in burned sites (9), which resembles closely to the thickness of human skin. The 5 mm probe gives information from the epidermis and the most superficial part of the dermis (6). Since these structures are firstly destroyed in thermal injury, all burns are likely to induce changes in the tissue water content. Accordingly, the water content measured with this probe was significantly lower in the full thickness (9S) burns compared to both the non-burned control skin at 24 hours (Fig. 4a) and the partial thickness (3S) burns enabling differentiation of partial and full thickness burns as soon as 8 hours post burn (Fig. 5 a).
The 15 mm probe gives information from the whole dermis. The dermal water content was higher in the 1S and 3S burn sites than in the control skin at 8 hours post injury relating to early edema formation in the skin. Moreover, the reduced dielectric constant in the 9S burns correlates to full thickness injury with no edema in the skin. Accordingly, the 15 mm probe could be used for differentiation of a full thickness burn from a partial thickness burn at 8, 24 and 72 hours (Fig. 5b).

The ROC analysis was used to find the dielectric constant values which would distinguish the partial and full thickness burns at different time points (Table 1). These burn depths (3S and 9S) were chosen for the analysis because of their great clinical significance. A deep burn was classified as a positive finding as it is clinically important to be able to determine which patients benefit of early surgery. The specificity with the superficial 5 mm probe was 100 % during the first 24 hours. Hence, the early ability to find those burns in this study which were not deep (true negatives) was excellent. Also, the positive predictive value (PPV) assessing the reliability of a positive finding with the 5 mm probe was greater than 94.7 % throughout the study. The PPV of the measurements performed with the dermal (15 mm) probe increased between 24-72 hours. This might correlate to the progressive tissue damage to deeper tissues, which is more clearly measurable with the 15 mm probe. Although these numbers can not be directly used in humans, these findings are of clinical importance. Early diagnosis of burn depth enables early treatment plans leading to either earlier discharge of patients or earlier surgical procedures thus shortening hospital stay.

The increase in blood flow in the adipose tissue after thermal injury seems to be related to a sustained fluid filtrate after the resuscitation period, resulting in edema formation mainly located

in the adipose tissue (29). The relative increase of tissue water in subcutaneous fat has been found to be as high as 434 % after a full thickness injury in sheep (29). In the present study, information from the subcutaneous fat was obtained by using the 30 mm probe with a sensitive depth of 5 mm (6). This, however, represents only about the superficial 2/3 of the fat thickness in these pigs. Still, all burn sites presented a dielectric constant almost twice as high as in the non-burned control site during the whole study period (Figures 2c, 3c and 4c). Accordingly, all burns induced subcutaneous edema regardless of burn depth. Therefore, the deep 30 mm probe can not be used to differentiate burns of different depths but it gives information of the edema in the subcutaneous fat. In order to receive information of the total fat layer, an even larger probe would be needed. However, it is unlikely that it would give any additional information regarding actual burn depth.

In practise, differentiation between partial and full thickness burns could be done by using the 5 and 15 mm probes together. Partial thickness burns result in significantly higher dielectric constants than full thickness burns with both probes (Fig. 5a and b). This can be secured with the finding that the dielectric constant measured with the 5 mm probe is significantly lower in the full thickness burn compared to that of non-burned skin (Fig. 4a), whereas there is practically no difference between the dielectric constant in partial thickness burns and the non-burned skin (Fig. 3a).

There were some limitations in the study. First, the number of the animals was rather small. However, the purpose of this pioneer study was to examine whether the dielectric measurements could provide specific information of burns of different depths, and this goal was achieved even with a low number of animals. Secondly, in order to avoid the problem of multi-testing, only burns of three different depths from the original histological study (9) and 4 different time points were chosen for the study. Thirdly, the threshold values for the dielectric constants presented in this study apply only for this animal model and can not be directly adapted to humans.

In conclusion, the dielectric measurements provide a useful tool for burn wound research to be used in clinical trials with special interest of early burn depth determination.

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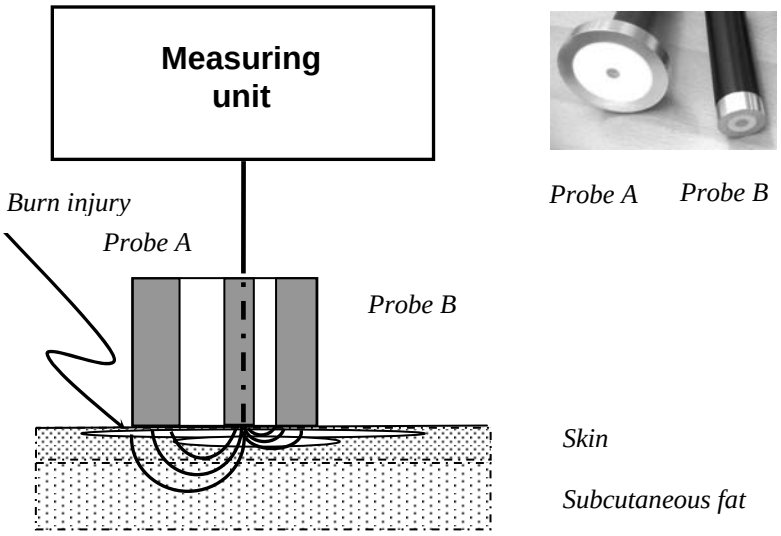


Figure 1. Schematic illustration of the dielectric measurement with burn injury and induced electrical field in skin and subcutaneous fat by bigger (A) or smaller probe (B), not in scale.

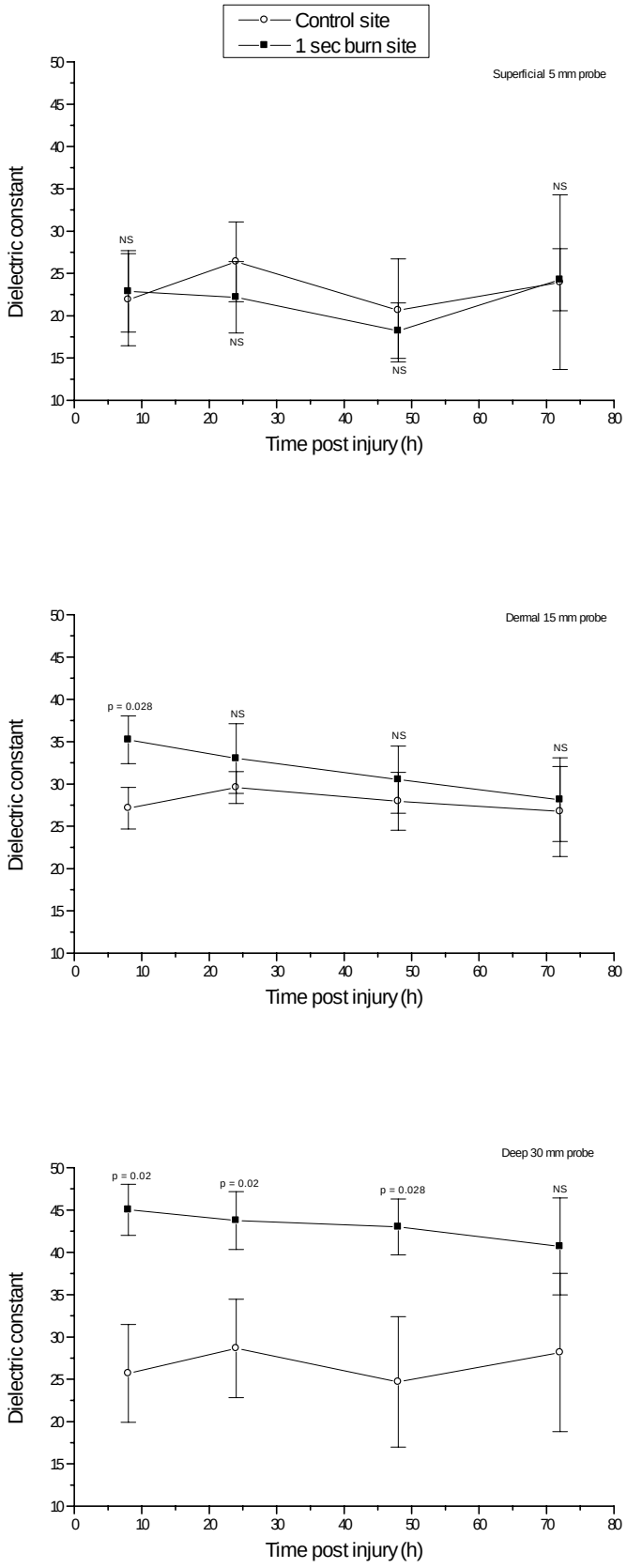
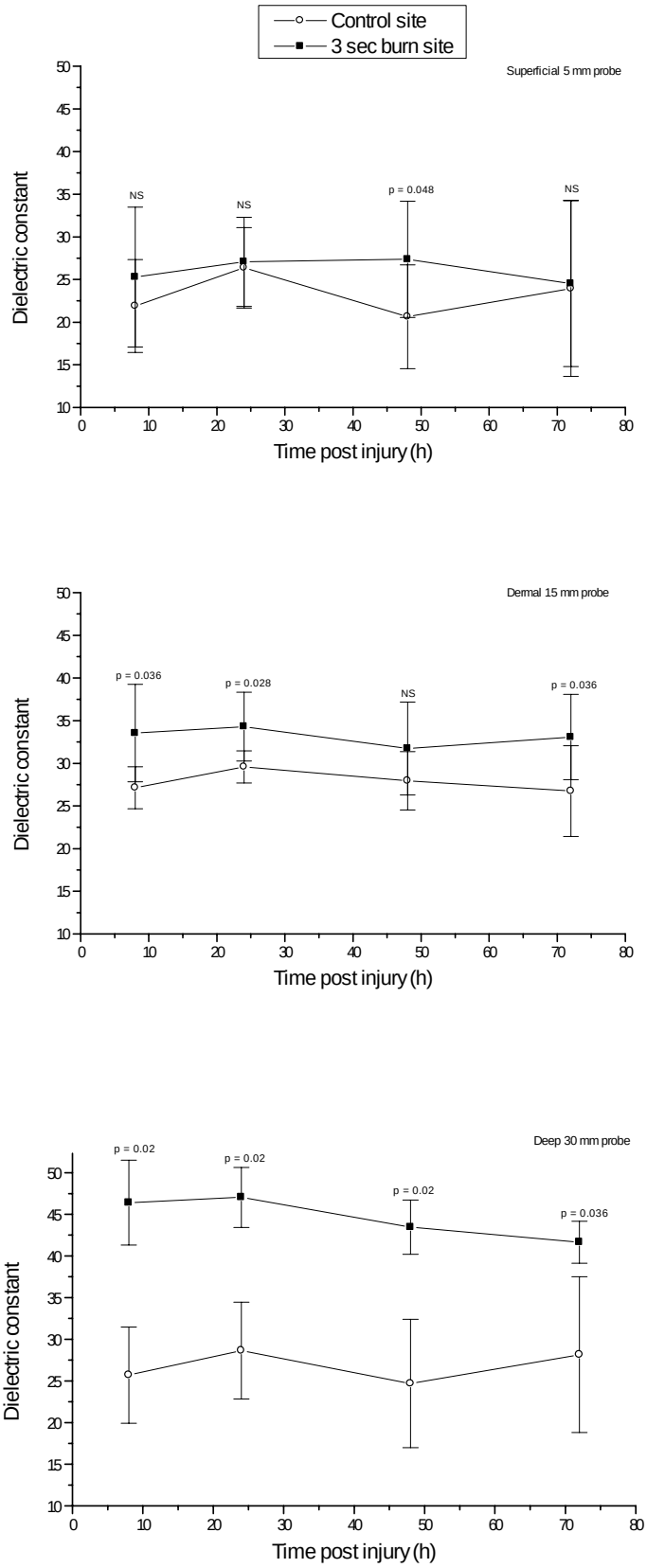
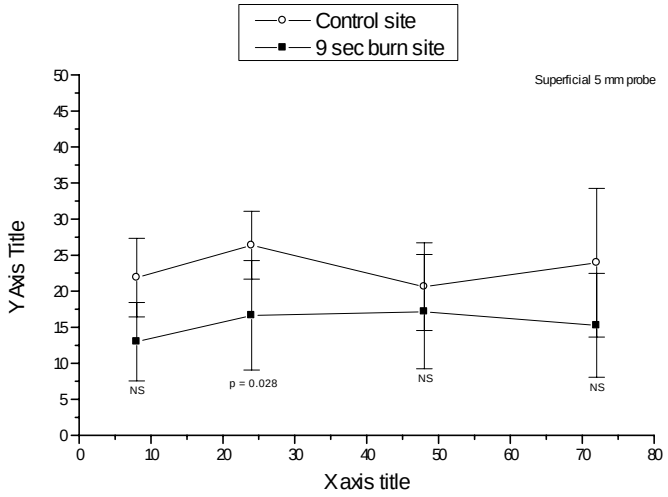
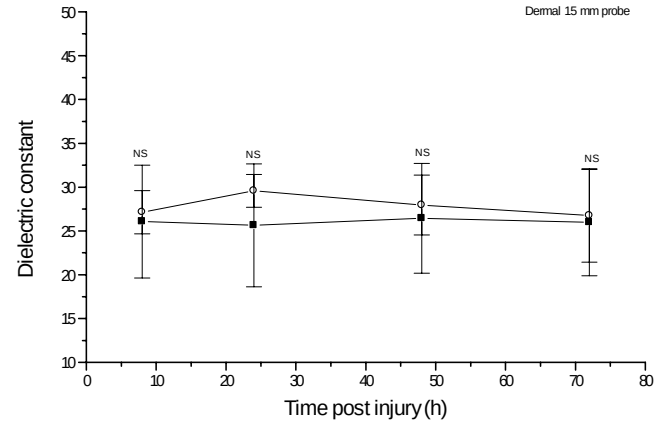


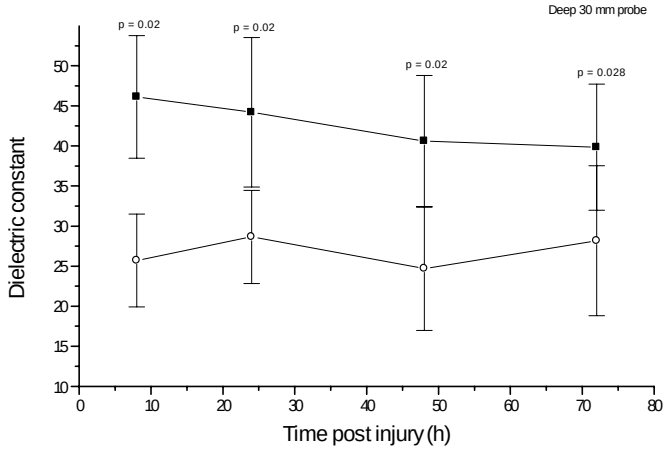
Figure 2a-c. The mean dielectric constant in the 1 second burns and the control site measured with the (a) 5 mm, (b) 15 mm and (c) 30 mm probes with the respective p-values.



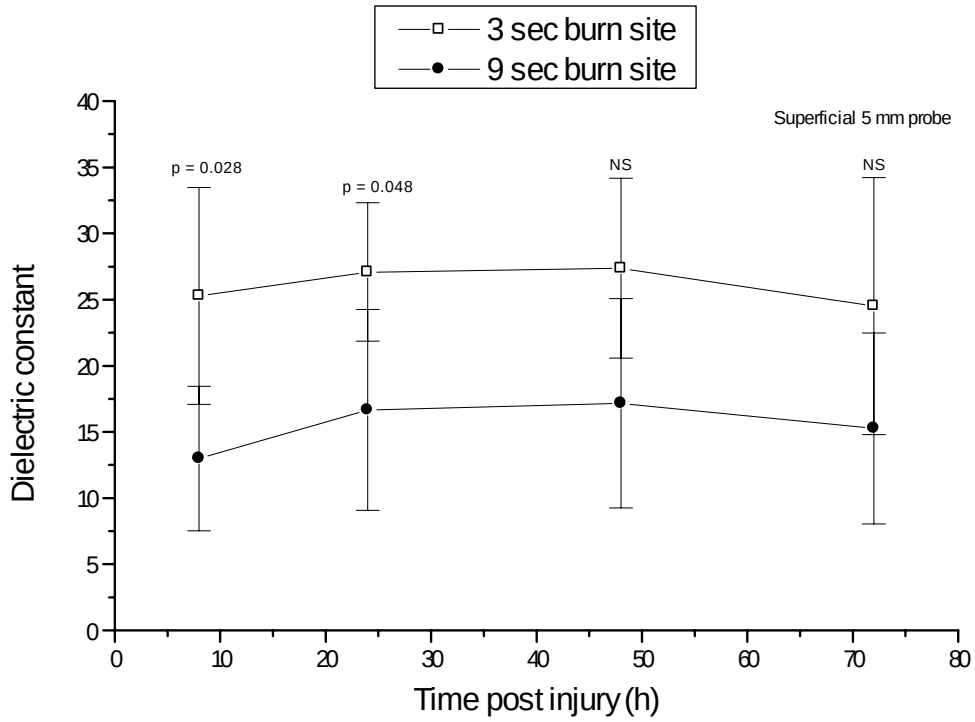
Figures 3a-c. The mean dielectric constant in the 3 second burns and the control site measured with (a) 5 mm, (b) 15 mm and (c) 30 mm probes with the respective p-values.

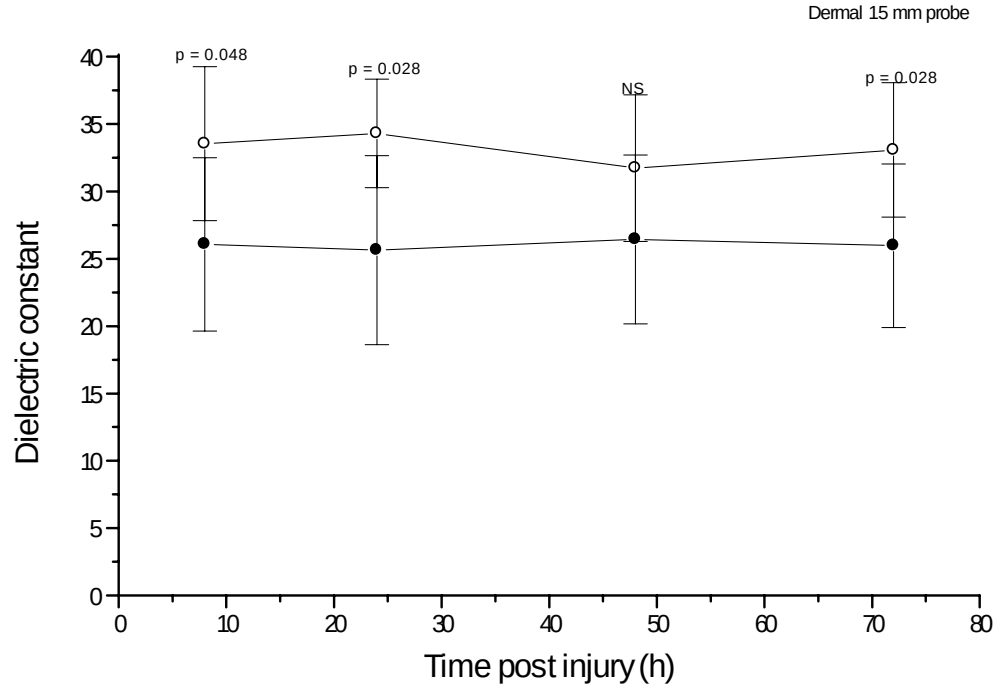






Figures 4a-c. The mean dielectric constant in the 9 second burns and the control site measured with the (a) 5 mm, (b) 15 mm and (c) 30 mm probes with the respective p-values.


3 sec burn site
9 sec burn site
Superficial 5 mm probe
p = 0.028
p = 0.048
NS
NS
Dielectric constant
Time post injury (h)
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80


Dermal 15 mm probe
p = 0.048
p = 0.028
NS
p = 0.028
Dielectric constant
Time post injury (h)
0
5
10
15
20
25
30
35
40
0
10
20
30
40
50
60
70
80

Figures 5a and b. Comparison of the mean dielectric constants in the 3 and 9 second burn sites measured with the (a) 5 mm and (b) 15 mm probes with the respective p-values.

Probe	Time (h)	DC	Sensitivity (%)	Specificity (%)	AUC	PPV (%)	NPV (%)
5mm	8	13	70	100	0.92	100	59
(superficial)	24	19	70	100	0.88	100	59
	48	20	80	90	0.85	99	33
	72	19	90	80	0.82	95	67
15 mm	8	29	80	80	0.82	94	50
(dermal)	24	26	70	100	0.88	89	10
	48	28	80	80	0.78	94	50
	72	28	80	90	0.86	97	53

DC = dielectric constant resulting in the best cutting point between 3 and 9 second burns
AUC = area under the curve
PPV = positive predictive value
NPV = negative predictive value

Table 1. Results of the ROC analysis. The dielectric values giving the highest accuracy of the method for specificity and sensitivity to distinguish the 3 second burns from the 9 second burns with the 5 and 15 mm probes are presented together with the respective values of AUC, PPV and NPV.

IV

Red blood cell and tissue water content
in experimental thermal injury

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Summary

Edema formation and changes in local blood flow are known phenomena in burns. The relationship between these two is not clearly described. The aim of this study was firstly to examine both the contents of red blood cells and tissue water in skin and subcutaneous fat after experimental burns of different depths in pigs and secondly confirm our recent findings of the increased dielectric constant of skin and subcutaneous fat reflecting considerable edema formation especially in fat after thermal injury.

Methods: Superficial, partial and full thickness contact burns were created to pigs and followed for 24 hours. Radioactive Cr-51 labelling of red cells was used to estimate the number of red cells in tissue and the absolute amount of water was determined by lyophilisation.

Results: A decreased number of labelled red cells in skin and an increase in tissue water in subcutaneous fat were found regardless of burn depth. The highest water amount in fat was found in the partial thickness burns.

Conclusion: All burn depths resulted in a diminished number of labelled red blood cells in skin and a significant increase in the absolute water amount in subcutaneous fat at 24 hours post injury. The findings in fat support our recent findings of highly elevated dielectric constants measured by the new in vivo method of dielectric measurements.

Keywords: burn; edema; red blood cell; blood flow; lyophilisation

Introduction

Thermal injury induces edema formation and changes in local blood volume and blood flow. The zone of stasis plays an integral role in the development of the final depth of the injury (1). The deep dermal vascular plexus gives blood supply to the skin adnexes, which are the main source of ceratinocytes needed for the re-epithelisation of superficial burns (2). Hence, progressive reduction of dermal blood supply has an effect on the final depth of the burn wound. Diminished blood supply in the reticular dermis lowers the tendency of the burn wound to heal spontaneously leading to excision and grafting.

Edema formation has a deleterious effect on the oxygen and nutrient delivery to the wound (3). Edema in burned tissue increases the risk of infection due to the lowered PO_2 values which might progress a partial thickness burn to a full thickness burn (3). Leape found that 86 % of the final increase in water content in full thickness burns in rat skin was accomplished within the first 5 minutes post injury (4). Edema formation consisted of a rapid phase during the first hour post burn and of a more gradual increase occurring during the next 12-24 hours. Deep burns in sheep create less edema than the superficial ones (5,6). The edema in deep burns reabsorbs more slowly, suggesting a continued vascular or lymphatic occlusion leading to a smaller portion of vessels capable for fluid exchange (5).

The relationship between water and red cell contents in tissue after burn injury is not clearly described. The water content in different depths of tissue after experimental thermal injury has been recently measured with the dielectric measurements (7). It was found that all burn depths induced a pronounced increase in the water content in the subcutaneous fat and that full thickness burns had lower tissue water content in whole dermis than the partial thickness burns.

The aim of this study was to examine both the contents of red blood cells and absolute tissue water in skin and subcutaneous fat after experimental burns of different depths in pigs and to verify the findings of our previous study (7) where highly increased dielectric constant was found in fat relating to increased tissue water.

Materials and methods

Experimental protocol

The study was approved by the Institutional animal care and use committee of the University of Kuopio, Finland. Five female Finnish landrace pigs (28-38 kg) were premedicated, cannulated, anaesthetized and monitored as described previously (6). Animals received 50 % glucose infusion which was adjusted to maintain normoglycaemia (blood glucose 5-7 mmol/l). Normovolaemia was maintained with Ringer's acetate (Ringersteril, Baxter, Vantaa, Finland) according to CVP 4-7 mmHg. As the total surface area burned was very small (1.2 – 2.2 %), no actual fluid resuscitation due to the burn was indicated.

Histologically confirmed superficial, partial thickness and full thickness contact burns were created with a brass plate heated up to 100° C on both sides of the ventral body of each pig by using 1 (1S), 3 (3S) and 9 (9S) second contact times, respectively, as described previously (6). A total of 10 burns of each depth were thus created in the experiment. One control site (CS) next to the burned areas served as a reference site (N = 5) in each pig. The burns and control sites were 4 x 4 cm in size with a 3 cm distance between each site. The follow-up time was 24 hours. The animals were kept sedated on the respirator throughout the study and killed with an overdose of saturated magnesium sulphate solution intracardially.

Chromium-51 labelling of pig red blood cells

Two venous blood samples (8 ml each) were collected from each pig prior to inflicting the burns. Both specimens were anticoagulated with 1.5 ml of acid citrate dextrose-A (ACD-A) solution. The samples were centrifuged at 3000 rpm for 10 minutes. Supernatant plasmas and buffy coats were discarded taking care not to remove any red blood cells (RBC). 2 MBq sodium chromate (Cr-51) solution (minimum 0.2 ml) was slowly added to each tube and the suspensions were incubated for 15 minutes at room temperature with continuous gentle mixing. Labeled cells were washed three times with isotonic saline and re-suspended in the original blood volume with isotonic saline (8). Aliquot (0.5 ml) of the labeled RBC suspension was taken for a radioactivity measurement while the rest of the suspension was reinfused in the animal through a vein cannula at 23.5 hours postburn. After 30 minutes (i.e. 24 h post burn) the animal was sacrificed with an intracardial injection of magnesium sulphate. Burn (N = 30) and control (N = 5) sites were excised surgically and the skin was sharply separated from the subcutaneous fat horizontally with scissors. Each tissue sample (N = 70) was again cut vertically in two pieces giving two tissue samples of skin and two samples of subcutaneous fat from each burn and control site for analysis.

Each sample (N = 140) was placed inside a test tube. Each test tube and tissue sample was weighed separately.

Determination of the number of labelled RBCs in tissue samples

Simultaneously with the collection of the blood samples for labelling, one blood sample (5 ml) was collected from each pig for the determination of the RBC count. The RBC count per ml of blood was determined by Sysmex K-4500 analyser (Sysmex, Kobe, Japan). Since a known volume of blood was drawn for labelling, total number of the labelled and reinfused RBCs could be estimated. The radioactivity of the tissues and the labelled RBC suspensions were counted with a 1480 Wizard gamma counter (Perkin Elmer, Turku, Finland) using a counting time of 30 min. The RBC suspensions were diluted 1:10000 with isotonic saline prior the measurement. Background-corrected counts for the diluted RBC suspensions were used to calculate counts of a single RBC. The number of the labelled RBCs per 1 g of tissue was determined by using background-corrected total counts for tissue samples and calculated counts of a single RBC.

Determination of tissue water content

Wet-weighed tissue samples were frozen at -70° C and lyophilisized with a Hetosicc CD52 freeze drying apparatus (Heto, Birkerød, Denmark). Lyophilisation was continued until the difference between 2 successive weightings was smaller than 0.002 g. The water content (g) was calculated as the difference between the wet and dry weight.

Statistics

The results are presented as mean $\pm$ 1SD. The non-parametric Wilcoxon signed rank test (2-tailed) was used for statistical analysis. A p-value <0.05 was considered statistically significant.

Results

Number of radioactively labelled red cells

The changes in the numbers of labelled red cells (LRC) per 1 g of wet tissue at different burn sites compared to the non-burned control sites are presented in Figure 1. The 1S, 3S and 9S burns caused a 46 %, 40 % and 54 % decrease in LRC in skin (Fig. 1a) at 24 hours post injury ($p = 0.028$ in all). The 1S and 3S burns caused a 15 % and 12 % increase in LRC in subcutaneous fat

(Fig. 1b) compared to the control site, but the changes were not statistically significant. The 9S burns caused a decrease of 19 % in LRC compared to the control site (CS) ($p = 0.028$) and was significantly lower than the 1S ($p = 0.01$). The difference between the 3S and 9S burn sites was non-significant ($p = 0.091$). At S1 and S9 burn sites the concentration of red cells in fat was higher than in skin ($p = 0.003$ and $p = 0.010$, respectively).

Total water amount in skin and subcutaneous fat

In the unburned control site the content of water in skin was 72.3 % and in subcutaneous fat 36.6 % (Table 1). While the water content in skin increased only slightly (Fig. 1a), the water content in fat (Fig. 1b) increased 64 %, 77 % and 67 % in the 1S, 3S and 9S burn sites, respectively, compared to CS ($p=0.028$ in all). The water content in the 3S burn site was higher than in the 1S ($p=0.003$) and in the 9S ($p=0.033$).

Discussion

The aim of this study was to determine the concentration of labelled red cells and the absolute amount of water in skin and subcutaneous fat 24 hours after creating burns of different depths in pigs and to verify our recent findings of burn-related elevation of dielectric constants in fat. The main findings were that burn trauma causes a significant decrease in the number of labelled red blood cells in skin and a significant increase in the amount of water in subcutaneous fat at 24 hours post injury regardless of the depth of the injury.

The pathologic changes in the burn wound circulation are progressive over the first 24 hours (9). Impairment of blood flow begins with events occurring in the microvasculature, including platelet microthrombus formation and vasoconstriction (10). During the first minute post injury, scald burn in rat has caused a 400 % increase in blood perfusion measured by laser Doppler (11). At 60 minutes the perfusion was about 100 % above the normal level. On the other hand, deep burns have decreased blood flow compared to the non-burned sites (9,12,13). Similar results were found in deep flame burns using cytometry in sheep, where skin blood flow was also decreased during a 72-hour-study (14). However, in the same study a biphasic reaction in blood flow was found in subcutaneous fat, where the initial decrease in blood flow was followed by a later hyperaemic phase on the third day post burn (14). In the present study, the number of radioactive red cells in skin was lower at all depths of burns than in the non-burned control sites at 24 hours post injury. This supports previous reports where in severely burned areas the impairment of blood flow ensued within a couple of hours and was delayed for up to 16-24 hours in less severe regions (15). Our study also demonstrated that the 9 second burn caused a significant decrease in the

number of labelled red blood cells in fat, which is in accordance with the findings of Ferguson et al in guinea pigs (13). Histologically, these 9 second burns have previously been found to be deep, full thickness burns (6). The decreased number of red cells in fat in these deep burns is in accordance with both the burn trauma extending down to the fat causing vascular thrombosis and possibly the increased amount of edema, which results in a decreased number of red cells per 1g of tissue.

Deep scald burns in sheep have less swelling than superficial ones (5,7,16). Even though the temporal development of the swelling process might be identical in both burns, the reabsorption of fluid is slower in deep burns suggesting continued vascular or lymphatic occlusion (5). In sheep, edema has been found to be maximal between 12-18 hours post injury and at 24 hours reabsorption was already occurring (5). In our study with pigs, we found the highest amount of water in skin in the 9S site. Although the finding was not statistically significant, it might reflect the previous finding of the slow reabsorption process described in deep burns (5).

The greatest changes in tissue water contents were found in subcutaneous fat. Compared to the non-burned control site, the superficial (1S), partial thickness (3S) and full thickness (9S) burns caused a 64 %, 77 % and 67 % increase in the absolute amount of water, respectively. In experimental burns in pigs, the recent dielectric measurements have also indicated a marked increase in tissue water content in subcutaneous fat regardless of burn depth at all time points in a 72-hour-follow-up study (7). When performed with 300 MHz, the dielectric measurement specifically gives information about the amount of both bound and free water (= total water) in tissue (17). It was found, that partial and full thickness burns could be differentiated during the first 24 hours according to their edema formation characteristics (7). A pronounced increase in the water amount in fat has also been described previously in 40 % total body surface area full thickness burns in sheep (14). In superficial burns only the papillary vascular plexus is destroyed. Most of the blood circulation in the skin is preserved making it the likely source of the edema fluid. When the burn depth increases, the circulation of both the papillary and reticular dermal plexuses are increasingly deteriorated. Thus, the remaining patent blood vessels are in the subcutaneous fat, explaining the increased fat edema especially in partial thickness and deep burns.

In conclusion, all burn depths resulted in diminished number of labelled red blood cells in skin and a significant increase in the absolute water amount in subcutaneous fat at 24 hours post injury. The findings in fat support the previous findings of highly elevated dielectric constants measured with the dielectric measurements (7).

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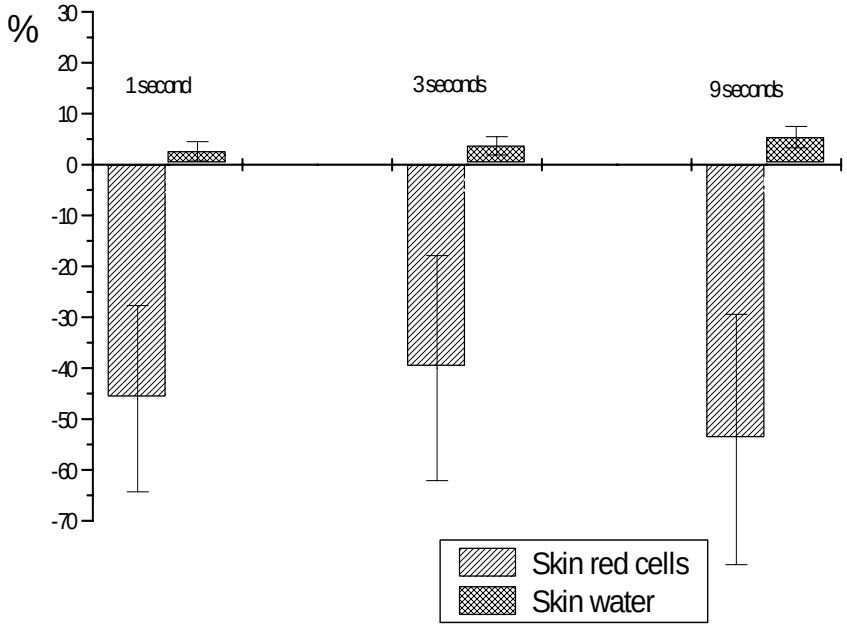
a



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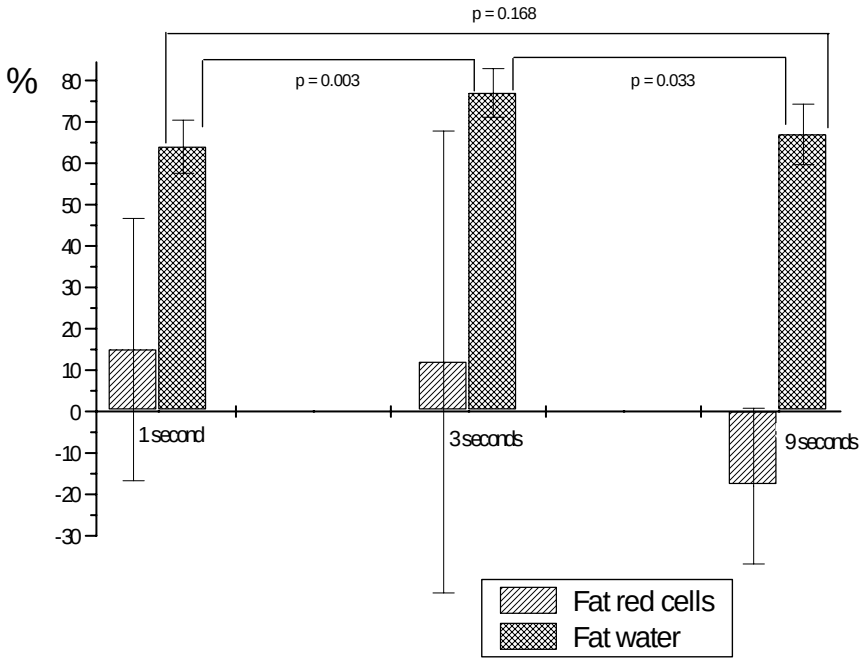

Figure 1a and b. The changes (mean $\pm$ SD) in the amount of labelled red cells and tissue water (a) in skin and (b) subcutaneous fat at different burn sites. Values express the percentual changes compared to the non-burned control site.

	SKIN	FAT
CS	72,3	36,6
1S	74,2	59,9
3S	75,0	64,8
9S	76,2	61,1

Table 1. Water amount (%) in lyophilisized skin and subcutaneous fat for the control site (CS) and different burn sites (1S, 3S, 9S).