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Biochemical markers for early detection of superficial pressure ulcers

PROEFSCHRIFT

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Debbie Bronneberg

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prof.dr.ir. F.P.T. Baaijens

Copromotoren:
dr. C.V.C. Bouten
en
dr.ir. C.W.J. Oomens

voor Bart
&
mijn ouders

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Summary

Pressure ulcers are areas of soft tissue breakdown resulting from sustained mechanical loading of the skin and underlying tissues. They are painful, difficult to treat, and represent a burden to the community in terms of health care and finances. The prevalence of pressure ulcers is unacceptably high and varies from 13% in hospitalized patients to 35% in institutions for the physically handicapped. This high prevalence is partly due to limited risk assessment. Currently, risk assessment is mainly performed by questionnaires or scales like the Norton, Braden, and Waterlow scale. Most risk assessment scales are based on expert opinion or literature review and have a limited scientific background. Furthermore, they do not predict the degree of susceptibility with the required accuracy and indeed, up to 30% of the hospitalized patients with pressure ulcers are still misclassified. Accordingly, patients with high risk might not receive adequate preventive measures.

The Dutch National Prevalence Survey demonstrated that grade I ulcers, the first stage of superficial ulcers in the skin, accounted for approximately 50% of the prevalence of pressure ulcers. Grade I ulcers are classified as non-blanchable erythema (NBE) of intact skin. Currently, the transparent disk method is commonly used for visual observation of NBE, in which the disk is pressed into the erythematous tissue. If the skin under this disk does not blanch, it is regarded as a grade I ulcer. The mentioned technique aims for measuring the consequences of 'harm' already done to the skin (i.e. inflammation). Whereas pressure ulcer prevention should aim for earlier detection of developing ulcers. Improved pressure ulcer risk assessment is, therefore, necessary. A first step in developing a new risk assessment tool is directed towards the detection of skin reactions that precede grade I ulcers. Cytokines and chemokines are of interest for pressure ulcer detection, since they are known to mediate inflammatory responses. The present thesis focuses on cytokines and chemokines as biochemical markers for early detection of superficial pressure ulcers.

A loading device has been developed to study the damaging effects of mechanical loading on an *in vitro* model of the epidermis. The commercially available EpiDerm cultures were employed as human epidermal equivalents. The general morphology of the EpiDerm cultures is comparable to human epidermis. Various degrees of epidermal damage were obtained by increasing either the magnitude or duration of loading.

Cytokines and chemokines, such as IL-1α, IL-1RA, TNF-α, CXCL8/IL-8, CCL20/MIP-3α, CCL2/MCP-1, and CXCL1/GRO-α, were evaluated as biochemical markers for mechanically-induced epidermal damage using this custom-built loading device.

IL-1α, IL-1RA, IL-8, and TNF-α were shown to be released as a result of mechanical loading. MIP-3α, MCP-1, and GRO-α could, however, not be measured. The release profiles of cytokines and chemokines were studied at various magnitudes and durations of mechanical loading. After 24 hours of loading with pressures ranging between 0 and 200 mmHg, an increase in the release of IL-1α, IL-1RA, IL-8, and TNF-α was observed at 75 mmHg and beyond. At the relatively low pressure of 75 mmHg, structural epidermal damage could barely be detected. So there seems to be a threshold for these markers, beyond which visible tissue damage occurs. Furthermore, as early as 1 hour after loading with 150 mmHg and before the onset of structural damage, the levels of IL-1α, IL-1RA and IL-8 were raised. Therefore, these markers are suitable for early detection of mechanically-induced epidermal damage in an *in vitro* setting. On the other hand, TNF-α seemed less suitable for early damage detection, since this marker was only raised when the first signs of structural damage were already apparent. Furthermore, the amount of TNF-α was very small and could hardly be detected using a 'high sensitive' immunoassay technique.

Urinary incontinence is widely recognized as an important risk factor for pressure ulcer development, although scientific proof was lacking. Therefore, biochemical markers, such as IL-1α and IL-1RA, were used to study the effect of synthetic-urine on the susceptibility of the epidermis to mechanical loading. An increase in epidermal damage, as well as in the release of IL-1α and IL-1RA was observed when both synthetic-urine and pressure were applied. Although these findings are obtained *in vitro*, they might indeed imply that urinary incontinent patients have a higher risk of developing pressure ulcers.

A clinical study was performed to determine whether IL-1α, IL-1RA, and IL-8 could also be detected in an *in vivo* setting. Sebutapes, adhesive films, were used to collect cytokines and chemokines from the skin surface in a non-invasive and painless way. Patients with a grade I ulcer at the sacrum, as well as patients without pressure ulcers participated in this study. For both patient groups, Sebutapes were applied to the sacrum as well as to the volar aspect of the left forearm (i.e. control site). Currently, only IL-1α could be detected using the Sebutape sampling method. For both patient groups, an increase in the absolute level of this cytokine was found at the sacrum compared to the volar aspect of the left forearm. This increase in IL-1α might be caused by sustained mechanical loading of the sacrum upon supine lying and sitting and was observed in spite of a high inter-subject variability in IL-1α. For patients with a grade I ulcer at the sacrum, the relative increase in IL-1α median ratio was higher when compared to the patient group without pressure ulcers. This result is promising and might again imply that a threshold is present for IL-1α beyond which visible tissue damage, as evidenced by NBE, occurs.

In conclusion, cytokines and chemokines seem promising biochemical markers for early detection of superficial pressure ulcers. A combination of different markers, rather than a single marker, is however required to sufficiently monitor the status of soft tissues (i.e. skin). Currently, the field of biosensor technology is emerging. A biosensor might eventually be developed that is able to monitor various biochemical as well as physiological markers to determine the patients risk for pressure ulcer development.

Chapter 1

General introduction

Parts of this chapter are based on D. Bronneberg and C. V. C. Bouten, *New tissue repair strategies*, in: Pressure ulcer research: current and future perspectives, Editors: Bader, D. L., Bouten, C. V. C., Colin, D., Oomens, C. W. J., Springer-Verlag, Book Chapter 20, pp 353-374, ISBN 3-540-25030-1 (2005).

1.1 Pressure ulcers

Pressure ulcers are areas of soft tissue breakdown resulting from sustained mechanical loading of the skin and underlying tissues (APUAP, 1998). Although pressure ulcers can occur anywhere on the body, they often develop adjacent to bony protrusions, e.g. in the trochanteric, ischial, heel, and sacral areas. These ulcers are painful, difficult to treat, and affect the quality of life of many young and elderly individuals. Furthermore, they represent a burden to the community in terms of health care and finances. Indeed, the annual costs of pressure ulcers in the Netherlands were estimated to be somewhere between 500 million and 2 billion dollars (Severens *et al.*, 2002).

Little is known about the pathophysiological responses to mechanical loading that trigger tissue breakdown (Bouten *et al.*, 2005). Various theories have been proposed to explain the onset of tissue damage. Ischaemia and impaired lymphatic drainage have been studied and generally confirm that sustained tissue loading will influence tissue perfusion and/or lymph flow, thereby affecting the transport of nutrients to and metabolic waste products away from cells within the tissue (Chang and Seireg, 1999; Herrman *et al.*, 1999). Although these theories might be appropriate for muscle tissue, which is metabolically more active than skin, they can only partly explain the onset of pressure ulcers and have to date not been fully verified. The same argument also applies to reperfusion injury mediated through oxygen free radicals (McCord, 1985, 1987; Peirce *et al.*, 2000). Lately, sustained cell deformation has been proposed as a trigger for cell damage, since existing histopathological data suggest a cellular origin to pressure ulcer development (Bouten *et al.*, 2001, 2003b; Berg and Rudolph, 1995). Indeed, studies demonstrated that cell deformation triggers a variety of effects, such as volume changes and cytoskeletal reorganization, which may be involved in early tissue breakdown (Bouten *et al.*, 2001, 2003b).

1.2 Risk assessment and ulcer detection

The prevalence of pressure ulcers is unacceptably high and varies from 15% in general hospitals to 24% in nursing homes (Halfens *et al.*, 2006). In our view, this high prevalence is partly due to limited risk assessment. At this moment, risk assessment is mainly performed by questionnaires or scales like the Norton (Norton, 1989), Braden (Bergstrom *et al.*, 1987), Waterlow (Waterlow, 1985), and CBO (CBO, 2002). Most risk assessment scales are based on expert opinion or literature review and have a limited scientific background (Schoonhoven *et al.*, 2002). Furthermore, Schoonhoven *et al.* (2002) demonstrated that risk assessment scales do not predict the degree of susceptibility with the required accuracy and indeed, up to 30% of the hospitalized patients with pressure ulcers are still misclassified. Accordingly, patients with high risk might not receive adequate preventive measures. Therefore, improved pressure ulcer risk assessment is necessary.

The European Pressure Ulcer Advisory Panel (EPUAP) defined four different pressure ulcer grades (table 1.1). Grade I ulcers, the first stage of superficial ulcers in skin,

accounted for approximately 50% of the prevalence in institutions in the Netherlands (Bours *et al.*, 2002). Grade I ulcers are classified as non-blanchable erythema (NBE) of intact skin (Defloor *et al.*, 2005). Currently, the transparant disk method is commonly used for visual observation of NBE, in which the disk is pressed into the erythematous tissue. If the skin under this disk does not blanch, it is regarded as a grade I ulcer (Halfens *et al.*, 2001; Vanderwee *et al.*, 2006). For individuals with darker skin types, it is more difficult to determine grade I ulcers. Alternative measures, such as skin temperature, edema, induration or skin hardness might be used in these individuals. Nonetheless, Rosen *et al.* (2006) demonstrated that patients with darker skin types are still less likely to have grade I ulcers identified compared to patients with lighter skin types. More importantly, the above mentioned technique is designed to assess the consequences of 'harm' already done to the skin (i.e. inflammation). Whereas pressure ulcer prevention should aim for earlier detection of developing ulcers. Improved pressure ulcer risk assessment is, therefore, necessary. A first step in developing a new risk assessment tool is directed towards the detection of skin reactions that precede grade I ulcers.

Grade I	Non-blanchable erythema of intact skin. Discolouration of the skin, warmth, edema, induration or hardness may also be used as indicators, particularly on individuals with darker skin.
Grade II	Partial thickness skin loss involving epidermis, dermis, or both. The ulcer is superficial and presents clinically as an abrasion or blister.
Grade III	Full thickness skin loss involving damage to or necrosis of subcutaneous tissue that may extend down to, but not through, underlying fascia.
Grade IV	Extensive destruction, tissue necrosis, or damage to muscle, bone, or supporting structures with or without full thickness skin loss.

Table 1.1: Pressure ulcer classification scheme (Defloor *et al.*, 2005).

Quintavalle *et al.* (2006) demonstrated that high resolution ultrasound might prove a useful tool for early detection of pressure ulcers. Ultrasound images obtained from patients 'at risk' of developing pressure ulcers showed areas of increased fluid content or edema at typical ulcer locations (i.e. heels, sacrum, ischial tuberosity). Other methods for pressure ulcer detection focussed on measuring interface pressures (Geyer *et al.*, 2001) or physiological responses of tissues to ischemia (Ferguson-Pell, 2005). The latter can be measured with a range of different techniques that provide information about the blood flow, tissue oxygenation, and blood content and oxygenation (Bader, 1990; Hagisawa *et al.*, 1994; Silver-Thorn, 2002; Wang and Vadgama,

2004). In contrast with high resolution ultrasound, in which early signs of tissue damage were measured, interface pressures and tissue responses to ischemia do not provide direct information on the damaged state of the tissue. Ideally, the tissue status should, thus, be monitored by objective markers that precede structural tissue damage.

The current thesis focusses on biochemical markers for early detection of superficial pressure ulcers. With respect to non-invasive measurements, these markers should be quickly released upon ulcer development and originate from cells in the superficial layer of the skin, the epidermis. Therefore, a detailed description of the skin, and in specific the epidermis, is given in section 1.3. Previous studies on pressure ulcers demonstrated that *in vitro* model systems are particularly useful in studying the damaging effects of well-controlled compressive loading regimes (Bouten *et al.*, 2001, 2003a; Breuls *et al.*, 2003; Gawlitta *et al.*, 2007). In the current thesis, an *in vitro* model of the epidermis (epidermal equivalent) was employed for performing mechanical loading studies. An extensive overview of the commercially available epidermal equivalents is, therefore, given in section 1.4. Furthermore, possible biochemical markers (i.e. cytokines and chemokines) for early detection of mechanically-induced epidermal damage, such as pressure ulcers, are described in section 1.5.

1.3 Skin

The skin is the largest organ of the human body, accounting for about 16% of total body weight and, in adults, presenting 1.2-2.3 m^2 of surface to the external environment (Junqueira *et al.*, 1995). It is composed of three layers (1) the epidermis, an epithelial layer, (2) the dermis, a layer of connective tissue, and (3) the hypodermis, a loose connective tissue layer that contains a pad of adipose cells (figure 1.1). The present thesis focusses on the epidermis.

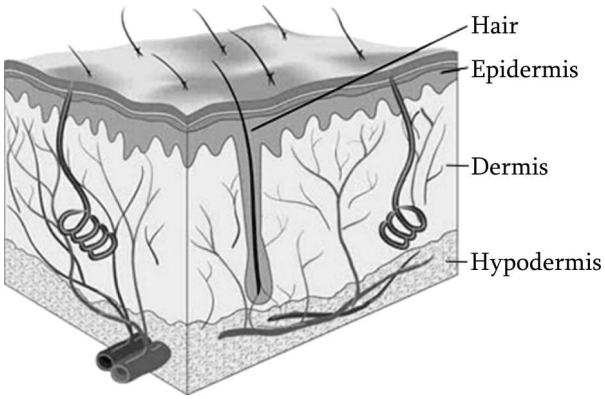

Figure 1.1: Schematic representation of the skin.

1.3.1 Epidermis

The epidermis is directly contiguous with the environment and acts as a permeability barrier. It prevents excessive water loss from the aqueous interior and protects internal tissues against mechanical insults, UV irradiation, the ingress of foreign chemicals and micro-organisms. The epidermis is avascular and mainly consists of keratin producing cells, which are called keratinocytes. Melanocytes, Langerhans cells and Merkel's cells are also present in the epidermis. The thickness of the epidermis varies between 400 and 600 μm for thick skin (palms of the hands and soles of the feet) and 75 and 150 μm for skin elsewhere on the body (Junqueira *et al.*, 1995). From the dermis outward, five layers can be distinguished: the stratum basale (basal layer), stratum spinosum (spinous layer), stratum granulosum (granular layer), stratum lucidum, and stratum corneum (cornified layer) (figure 1.2).

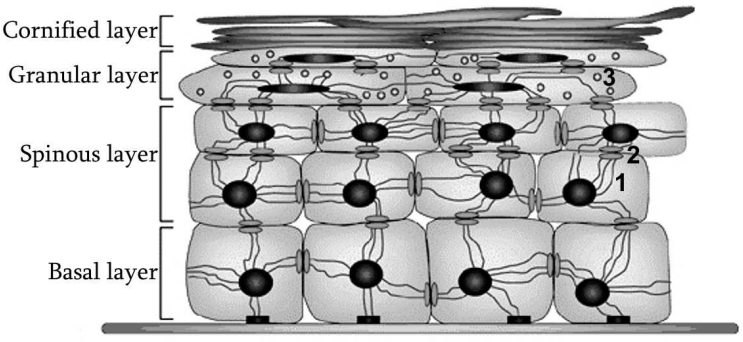

Figure 1.2: Schematic representation of the epidermis. The cells in the epidermis contain tonofilaments (1) and are connected by desmosomes (2). In the granular layer keratin granules are present (3).

Keratinocytes originate from the stratum basale and migrate through the superior layers of the epidermis, undergoing gradual differentiation until they reach the stratum corneum where they form a layer of dead, flattened, highly keratinized cells. These cells are continuously shed of the surface of the stratum corneum, a process which is called desquamation. Renewal of the epidermis under normal conditions occurs every 15 to 30 days.

The stratum basale is the deepest layer of the epidermis (figure 1.2). It consists of a single layer of dividing, undifferentiated, columnar or cuboidal shaped cells resting on the basal lamina at the dermal epidermal junction (Junqueira *et al.*, 1995). Hemidesmosomes help bind these cells to the basal lamina, whereas desmosomes in great quantity bind the cells in their lateral and upper surfaces. The stratum basale is characterized by intense mitotic activity and is responsible, in conjunction with the lower portion of the stratum spinosum, for constant renewal of epidermal cells. All cells in the stratum basale contain intermediate filaments (keratin).

The stratum spinosum is composed of several layers of cuboidal, polygonal cells

that slightly flatten when they progress toward the stratum granulosum (figure 1.2) (Junqueira *et al.*, 1995). These cells contain numerous tonofilaments that cross their cytoplasm and end at the desmosomes, by which the cells are connected. These tonofilaments play an important role in maintaining the cohesion among the cells and resisting the effects of abrasion.

The stratum granulosum is composed of 2 to 5 layers of flattened, polygonal cells (figure 1.2) (Junqueira *et al.*, 1995). The cytoplasm of these cells is filled with keratohyalin granules, which contain profilaggrin. The protein filaggrin plays a specific role in the aggregation of intracellular keratin filaments in the outermost layers of the epidermis. Another characteristic structure found in the cells of the stratum granulosum are the membrane coating granules (lamellar bodies), a small ovoid structure containing lamellar disks that are formed by lipid bilayers. These granules contain glycosaminoglycans and phospholipids, which are exocytosed into the space between the stratum granulosum and the stratum corneum where they are deposited in the form of lipid enriched membranes. Furthermore, cytolysis takes place in the upper layer of the stratum granulosum, which results in the accumulation of proteins (involucrin and keratolinine). This protein layer gives extra strength to the cells next to the intracellular keratin filaments. The stratum lucidum is mainly apparent in thick skin and is a translucent, thin layer of extremely flattened eosinophilic cells.

The stratum corneum is composed of 10-15 layers of dead, keratin-filled, squamous cell (corneocytes) that are surrounded by a matrix of lipid enriched membranes (figure 1.2) (Junqueira *et al.*, 1995). Elias and Williams (2000) described the stratum corneum as a two-compartment system which can be likened to a brick wall, composed of corneocytes (the 'bricks') and intercellular lamellar membranes (the 'mortar'). The lamellar membranes of the stratum corneum serve as an important sealing compound in the skin; these membranes actually form the skin barrier.

1.4 Epidermal equivalents

A wide range of tissue engineered epidermal equivalents are commercially available (Bronneberg and Bouten, 2005). The term tissue engineering refers to the application of the principles and methods of engineering and the life sciences toward the development of biological substitutes to restore, maintain, or improve function (Lee, 2000). Some of these epidermal equivalents are used as advanced therapeutic products for the treatment of burns and chronic wounds (e.g. venous and diabetic ulcers), while others are used as diagnostic products for topical irritation, corrosivity and other testing studies. Furthermore, these products differ with respect to the source of the cells and extracellular matrix (i.e. autologous, obtained from the patient; allogenic, obtained from a human donor; xenogenic, obtained from an animal donor), culture conditions, shelf life, and costs. An overview of the commercially available epidermal equivalents is given below.

Epicel, developed by Genzyme Biosurgery, is one of the oldest, autologous epidermal equivalents. It was first created in 1975, but has only been commercially available since 1988 (Bello *et al.*, 2001). It derives from, a small skin biopsy sample,

from which a cell suspension of epidermal keratinocytes can be obtained. This cell suspension is seeded and cultured on lethally irradiated 3T3 mouse fibroblasts. When the cultures reach confluence, the keratinocyte sheets are released with dispase and attached to a nonadherent gauze dressing (Green *et al.*, 1979; Bello *et al.*, 2001). Epicel provides permanent wound coverage and has been used to treat burns (O'Connor *et al.*, 1981; Gallico *et al.*, 1984), chronic leg ulcers (Hefton *et al.*, 1986), and pressure ulcers (Phillips and Pachas, 1994).

EpiDex, developed by Modex Thérapeutiques, is another autologous epidermal equivalent composed of a keratinocyte sheet (Ramos-Silva and de Castro, 2002). This epidermal equivalent does not rely on skin biopsies, but is generated from the patient's hair. Precursor cells for epidermal keratinocytes can be easily obtained from plucked scalp hair follicles and these cells retain a high proliferative capacity irrespective of the age of the donor follicle.

In general, engineered keratinocyte sheets are fragile, hard to handle, and display unstable attachment without dermal substrate. Fidia Advanced Biopolymers is attempting to improve these properties by providing a film of benzolyated hyaluronic acid as an additional dermal equivalent (Mansbridge, 2002). This biodegradable film, termed Laserskin, has periodic perforations through which the keratinocytes can migrate to reach the dermis of the host. ConvaTec also generated autologous keratinocyte sheets on porous films of hyaluronic acid (VivoDerm) (Ramos-Silva and de Castro, 2002). This epidermal equivalent has mainly been used for burns and chronic wounds, such as venous ulcers (Phillips, 1999; Sefton and Woodhouse, 1998).

Prunièras *et al.* (1983) rendered the culture of keratinocytes more physiological by raising them to the air-liquid interface. By doing so they obtained evidence of a more complete differentiation, as evaluated by morphological criteria. Under the electron microscope, several ultrastructural features reminiscent of those seen in situ could be identified, including tonofilaments, desmosomes, and cell-membrane thickening. Furthermore, the stratum corneum barrier function of the epidermal equivalent was found to be markedly improved by topical exposure to air.

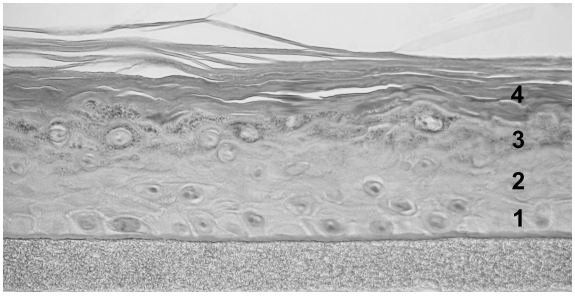

Figure 1.3: A cross-sectional image of an EpiDerm culture. The stratum basale (1), stratum spinosum (2), stratum granulosum (3), and stratum corneum (4) can be distinguished in this culture.

Keratinocytes can be raised to the air-liquid interface by culturing them on dermal

substrates or dermal equivalents. Episkin, developed by Episkin SNC, is generated by seeding keratinocytes on a dermal substrate composed of a thin bovine collagen type I matrix on which was a film of human collagen type IV (Ponec *et al.*, 2000, 2002). MatTek Corporation also uses dermal substrates to expose the keratinocytes to air and generate epidermal equivalents. EpiDerm is composed of human keratinocytes that are seeded on cell culture inserts coated with collagen (figure 1.3). SkinEthic, developed by Laboratoire SkinEthic, is also composed of human keratinocytes that are seeded on inert polycarbonate cell culture inserts. These three epidermal equivalents are currently used as diagnostic products for topical irritation, corrosivity, and other testing studies (Coquette *et al.*, 2003; Faller and Bracher, 2002; Gibbs *et al.*, 2002; Mansbridge, 2002; Ponec *et al.*, 2000, 2002; Netzlaff *et al.*, 2005).

1.5 Biochemical markers

The epidermis and its main constituent, keratinocytes, used to be thought of as merely a passive barrier between the external environment and internal organs (Uchi *et al.*, 2000). However, in the late 1970s it was found that keratinocytes can produce interleukin 1 (IL-1) and granulocyte-macrophage colony stimulating factor (GM-CSF). Discoveries like these led to the concept that keratinocytes are an important component of the skin immune system and actively mediate various kinds of biological responses (Bos and Kapsenberg, 1993; Uchi *et al.*, 2000). Keratinocytes influence a variety of physiological functions of the skin, including growth, differentiation, immune defense, inflammation, apoptosis, and wound healing. In unstimulated keratinocytes, inflammatory mediators are barely detectable. However, upon external stimulation keratinocytes secrete (pro-inflammatory) cytokines to activate both epidermal and dermal cells, and chemokines to attract leukocytes from the circulation to the inflammatory foci. Cytokines and chemokines, which precede inflammatory responses, are of interest for early detection of superficial pressure ulcers.

1.5.1 Cytokines

Cytokines represent a heterogeneous family of inducible glycoproteins produced by various cell types, which mediate local interaction and distant communication between cellular elements of immune and inflammatory responses (Corsini and Galli, 1998). Cytokines may act directly, as inducers and regulators of cell growth, division and differentiation, as stimulaters of cell movement and migration and as controllers of cellular function and interactions via induced changes in the expression of adhesion molecules and receptors for cytokines (Corsini and Galli, 1998).

IL-1 is a pluripotent, multifunctional cytokine that plays a key role in the initiation and development of inflammatory and immune responses (Uchi *et al.*, 2000). Keratinocytes constitutively produce IL-1α, IL-1β, and their competitive antagonist, interleukin 1 receptor antagonist (IL-1RA). Both IL-1α and IL-1β have similar biological activities, but keratincytes do not secrete these proteins simultaneously. Keratinocytes maintain prodigious stores of IL-1 in the cytoplasm which are normally lost

by desquamation of the stratum corneum. However, upon skin injury or even mechanical deformation of keratinocytes, a large amount of stored IL-1 is released and can trigger a rapid immune response (Lee *et al.*, 1997; Uchi *et al.*, 2000). IL-1 induces the production of other cytokines and chemokines such as interleukin 6 (IL-6), interleukin 8 (CXCL8/IL-8), tumor necrosis factor α (TNF-α), and GM-CSF as a mediator of the acute-phase response and its synthesis is induced by TNF-α, GM-CSF, and transforming growth factor β1 (TGF-β1). Il-1 also promotes differentiation of keratinocytes (Enk and Katz, 1992; Uchi *et al.*, 2000). IL-1RA is a competitive inhibitor of IL-1 and its activity is regulated by the ratio of IL-1RA to IL-1. Both IL-1α and IL-1β are synthesized as 31-kDa precursors that can be cleaved to mature 17-kDa molecules. IL-1α is biologically active in both precursor and mature forms, while IL-1β requires proteolytic cleavage by interleukin 1 converting enzyme (ICE) for full activity. In the human epidermis, the majority of IL-1 activity from keratinocytes involves IL-1α.

TNF-α is known to induce the expression of adhesion molecules and attracts neutrophils or lymphocytes to the skin (Groves *et al.*, 1995; Uchi *et al.*, 2000). In the epidermis, TNF-α is mostly produced by keratincytes and langerhans cells (Uchi *et al.*, 2000). The molecular weight of TNF-α under denaturing conditions is 17-kDa. The production of TNF-α by keratinocytes is increased by interferon γ (IFN-γ) and IL-1α, while TNF-α stimulates IL-1α and IL-8 production by keratinocytes.

1.5.2 Chemokines

Chemokines (approximately 8 to 14 kD) are small heparin-binding proteins that have chemotatic activity (Uchi *et al.*, 2000). They are divided into the CXC, CC, C, and CX3C subfamilies according to the positions of characteristic structure-determining cysteine residues within the N-terminal part of the proteins. Responses to chemokines are mediated by seven transmembrane-spanning G protein-coupled receptors. Keratinocytes synthesize many chemokines including members of the CC and CXC subfamilies and express some chemokine receptors that mediate the inflammatory or immune responses by attracting various kinds of leukocytes.

Basal cells in the epidermis constitutively express macrophage inflammatory protein 3 α (CCL20/MIP-3α), which attracts dendritic cells into the epidermis (Uchi *et al.*, 2000). Keratinocytes further produce monocyte chemotactic protein 1 (CCL2/ MCP-1). This chemokine may contribute to attracting eosinophils, T-cells, dendritic cells, and monocytes into inflamed skin lesions. Furthermore, upon stimulation with UVB irradiation or chemicals, keratinocytes produces CXCL8/IL-8 and growth-regulated protein precursor α (CXCL1/GRO-α), both of which are neutrophil attractants. Normal keratinocytes do not synthesize CXCL1/GRO-α and CXCL8/IL-8, but their production can be induced upon skin injury. In addition, CXCL1/GRO-α has the capacity to promote the proliferation of keratinocytes and thus participate in wound healing.

1.5.3 Epidermal damage markers

IL-1α and IL-RA have widely been used as screening parameters for measuring epidermal reactivity to various chemicals (Bernhofer *et al.*, 1999; Faller and Bracher,

2002). Lee (2000) and Takei *et al.* (1998), further, showed that mechanical straining of keratinocyte monolayers promotes the expression and release of IL-1α and IL-1RA. In addition, Wood *et al.* (1992, 1997) demonstrated an increase in TNF-α after tape stripping of the epidermis. IL-1α and TNF-α are, further, known to stimulate the release of chemokines such as CXCL8/IL-8, CCL20/MIP-3α, CXCL1/GRO-α, and CCL2/MCP-1 in epidermal cultures (Nakayama *et al.*, 2001; Tohyama *et al.*, 2001; Steude *et al.*, 2002; Wetzler *et al.*, 2000). These cytokines and chemokines might, therefore, be suitable markers for early detection of mechanically-induced epidermal damage, such as pressure ulcers.

1.6 Objective and Outline

The objective of the current thesis is to determine biochemical markers that are suitable for early detection of superficial pressure ulcers. In order to achieve this objective it is necessary to:

- Study the release profiles of biochemical markers in an *in vitro* model for pressure ulcer development.
- Study the effect of a pressure ulcer risk factor on the susceptibility of *in vitro* epidermis to mechanical loading using biochemical markers.
- Determine whether the identified biochemical markers can actually be measured *in vivo* in a clinical setting.

For these purposes, a loading device was developed to study the damaging effects of mechanical loading on a *in vitro* model of the epidermis (chapter 2). The commercially available EpiDerm cultures (MatTek Corporation, MA, USA) were employed as human epidermal equivalents in the present thesis. Various degrees of epidermal damage were obtained by either increasing the magnitude or duration of mechanical loading. Cytokines and chemokines (IL-1α, IL-1RA, TNF-α, CXCL8/IL-8, CCL20/MIP-3α, CCL2/MCP-1, and CXCL1/GRO-α) were evaluated as biochemical markers for mechanically-induced epidermal damage using this custom-built loading device. The release profiles of cytokines and chemokines were studied at various load magnitudes (chapter 3) and durations (chapter 4). Cytokines and chemokines (IL-1α, IL-1RA, TNF-α, CXCL8/IL-8) that were released before the onset of visible epidermal damage are of interest for early detection of pressure ulcers. IL-1α and IL-1RA were, subsequently, used as objective markers to provide experimental evidence for a relation between pressure ulcers and urinary incontinence (chapter 5). Urinary incontinence is widely recognized as an important risk factor for pressure ulcer formation, although scientific proof was still lacking. Finally, an *in vivo* study was performed to determine whether cytokines and chemokines can be identified in a clinical setting using a non-invasive detection method (chapter 6). This thesis closes with a general discussion about the presented work (chapter 7).

Chapter 2

An *in vitro* model system to study the damaging effects of epidermal loading

The contents of this chapter are based on D. Bronneberg, C. V. C. Bouten, C. W. J. Oomens, P. M. van Kemenade, and F. P. T. Baaijens (2006), *An in vitro model system to study the damaging effects of prolonged mechanical loading of the epidermis*, Annals of Biomedical Engineering; 34: pp 506-514.

2.1 Introduction

Prolonged mechanical loading of soft tissue can lead to tissue breakdown in the form of pressure ulcers (APUAP, 1998). Pressure ulcers are painful, difficult to treat and represent a burden to the community in terms of health care and cost (Bouten *et al.*, 2003b). In the skin these ulcers are presented by the following sequence of stages: blanchable hyperemia, non-blanchable hyperemia, ecchymosis (haemorrhagic spot), and finally tissue necrosis (Peirce *et al.*, 2000). Skin tissue can be conceived as consisting of two distinct layers: the avascular epidermis and the vascular dermis (Junqueira *et al.*, 1995; Wang and Sanders., 2005). Most of the research on the aetiology of skin pressure ulcers has focused on the effects of mechanical loading on the dermis, as its vasculature is considered to play a key role in the onset of these ulcers (Wang and Sanders., 2005). Various studies have shown that sustained skin loading can reduce the blood flow and/or lymph flow, thereby obstructing the transport of nutrients (e.g. oxygen) to and metabolic waste products away from cells within the tissue (Bouten *et al.*, 2003b; Chang and Seireg, 1999; Herrman *et al.*, 1999). The restoration of blood flow after load removal can also cause tissue breakdown through the excessive formation of oxygen-derived free radicals (McCord, 1985, 1987; Peirce *et al.*, 2000). However, these factors can only partly explain the onset of pressure ulcers in the avascular epidermis, since the energy metabolism of this skin layer is shown to be functionally anaerobic (Ronquist *et al.*, 2003). Recent studies on soft tissue breakdown focus on the role of sustained cell deformation in the damage process, since existing histopathological data suggest a cellular origin to pressure ulcer development (Bouten *et al.*, 2001, 2003b; Berg and Rudolph, 1995). Cell deformation is known to trigger a variety of effects, such as local membrane stresses, volume changes, and cytoskeletal reorganization, which may be involved in early tissue breakdown (Bouten *et al.*, 2001, 2003b). Similar effects can be expected for the epidermis. Because it is impossible to study cellular responses to *in vivo* loading independent of other factors, an *in vitro* model of the epidermis under mechanical loading is used in the present study.

Various *in vitro* models of the epidermis (epidermal equivalents) are commercially available (Mansbridge, 2002; Ponec *et al.*, 2002). In general, these engineered epidermal equivalents consist of normal, human-derived keratinocytes, which have been cultured on porous, flexible membranes of cell culture inserts at the air-liquid interface to form a multilayered, differentiated model of the human epidermis (Rosdy and Clauss, 1990). The stratum corneum, the protective skin barrier, has also been demonstrated in these engineered epidermal equivalents (Ponec *et al.*, 2002). The primary function of the stratum corneum *in vivo* is to retard evaporative water loss from the aqueous interior. Furthermore, the stratum corneum protects against mechanical insults and the ingress of foreign chemicals and microorganisms (Elias and Friend, 1975; Leveque *et al.*, 2002). Mainly due to their barrier capacity, these epidermal equivalents are particularly used as screening models for testing skin irritation of chemicals, as alternatives to animal testing (Coquette *et al.*, 2003; Faller and Bracher, 2002; Faller *et al.*, 2002; Gibbs *et al.*, 2002; Welss *et al.*, 2004). Nevertheless, they could also be used to investigate the epidermal responses to mechanical stimuli. In

the present study, EpiDerm (MatTek Corporation, Ashland, MA, USA) has been used as an epidermal equivalent to investigate the effects of sustained loading. This epidermal equivalent was chosen for its *in vivo* like morphology and growth characteristics, which are uniform and reproducible (Cannon *et al.*, 1994).

The aim of the present study was to characterize the effects of clinically relevant mechanical loading regimes, varying in magnitude and duration, on the damage progression in engineered epidermal equivalents (EpiDerm). For this purpose an *in vitro* loading device was developed in which six epidermal equivalents could be loaded simultaneously. Within the device environmental conditions, such as air temperature, humidity, and CO_2, can be controlled. The damage progression due to prolonged mechanical loading was determined by various endpoint measurements. First, tissue damage was assessed by histological examination. Second, the viability of the epidermal equivalents was determined. And third, the release of a pro-inflammatory mediator, interleukin 1 α (IL-1α), was measured as a marker of the onset of tissue damage.

2.2 Materials and methods

2.2.1 Engineered epidermal equivalent

A commercially available human epidermal equivalent, EpiDerm (EPI-200, MatTek Corporation, Ashland, MA, USA), was used as an *in vitro* model of the epidermis in this study. This model (diameter=8 mm and thickness$\approx$150 μm) consists of human-derived epidermal keratinocytes, which have been cultured on porous, flexible membranes of cell culture inserts at the air-liquid interface to form a multilayered, differentiated model of the human epidermis (Cannon *et al.*, 1994). The porous, flexible membrane of the EpiDerm cultures is situated 1 mm above a rigid culture plate due to the dimensions of the cell culture inserts.

Upon receipt, the EpiDerm cultures were transferred to 6-well plates containing 900 μl/well of hydrocortisone-free maintenance medium (EPI-100-MM-HCF, MatTek Corporation, Ashland, MA, USA), according to recommendations from the supplier. After overnight culture at 37°C and 5% CO_2 the cultures were transferred to fresh medium and directly used in a loading experiment. Four different batches of EpiDerm cultures were used in this study.

2.2.2 Loading of epidermal equivalents

EpiDerm cultures were subjected to sustained static loading, using a custom-built loading device, which was placed in an incubator at 37°C and 5% CO_2 (figure 2.1). This loading device consists of a stainless steel frame on which a 6-well plate containing EpiDerm cultures is placed, and six polycarbonate indenters (diameter=5 mm) to load the cultures simultaneously in a direction perpendicular to their air-exposed surfaces. Various weights can be put on top of these indenters to apply specified pressures to the cultures. In this study, clinically relevant pressures of 6.7 kPa (50 mmHg)

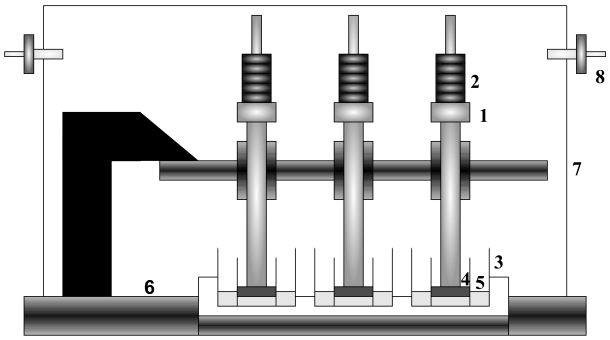

Figure 2.1: Schematic image of the custom-built loading device. In this representation (1) indicates an indenter, (2) the weights, (3) a 6-well plate, (4) an EpiDerm culture, (5) culture medium, (6) the stainless-steel frame, (7) the plastic cover, and (8) an air-filter.

(low-pressure: LP) and 13.3 kPa (100 mmHg) (high-pressure: HP) were applied for periods of 2 and 20 h. The cultures loaded for 2 h were statically cultured for an additional 3 h before damage analysis. This culture time was employed to increase the probability of measuring a significant release of the pro-inflammatory mediator, IL-1α. A loading time of 20 h was imposed to increase the probability of inducing visible tissue damage. Indeed, previous porcine studies demonstrated that skin breakdown only occurred at comparable pressures after relatively long loading periods (Daniel *et al.*, 1981). Furthermore, the pressures, which are imposed in the present study, can be experienced by humans at the skin-mattress interface while lying in bed (Wang and Sanders., 2005; Whittemore, 1998). Unloaded cultures (control: C) and cultures loaded with a small plate alone (diameter=5 mm) of negligible weight (plate control: PC) were used as control groups in this study. One batch of EpiDerm cultures was used for each loading experiment (C group (n=3), PC group (n=3), LP group (n=3), and HP group (n=3)). Furthermore, both the short- and long-term loading experiments (i.e. short: 2 h loading and 3 h static culture, long: 20 h loading) were repeated. In table 2.1 the layout of the loading experiments is displayed. After the

Batch	**Load duration**	**Load magnitude**
1-2	Short-term	C, PC, LP, HP
3-4	Long-term	C, PC, LP, HP

Table 2.1: Experimental layout. The loading regimes, varying in duration (short-term and long-term) and magnitude of loading (C, PC, LP and HP groups), are indicated for the different batches of EpiDerm cultures (batch 1-4).

short- and long-term loading experiments the culture supernatant underneath the

EpiDerm cultures was collected from each well and stored at -80°C for extracellular IL-1α analysis. The cultures were washed twice with the provided phosphate buffered saline (PBS, MatTek Corporation, Ashland, MA, USA) and either fixated for histological examination or used in a viability assay. These damage-assessment techniques are described below.

2.2.3 Damage assessment

Histological examination

The EpiDerm cultures (n=1 per experimental group) were fixed in 10% phosphate-buffered formalin and processed for conventional paraffin embedding. Sections (5 mm) were cut, deparaffinized, and stained for light microscopic examination. For the assessment of structural tissue damage a hematoxylin and eosin staining (H&E) was used. In addition, sections were stained with pyronine Y to specifically determine keratinocyte damage by a decrease in cytoplasmic RNA. For this purpose, sections were incubated in a fresh pyronine Y-staining solution (i.e. 0.1% pyrinine Y (Fluka chemie GMBH, Buchs, CH) in 0.2 M sodium acetate buffer, pH 4.0) for a period of 20 min at room temperature. The sections were subsequently washed three times in water, air dried, and embedded in Depex mounting medium (Gurr BDH lab. Supplies, Poole, UK).

Viability measurement

The viability of the EpiDerm cultures (n=2 per experimental group) was determined by a colorimetric MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide) assay (MTT-100, MatTek Corporation, Ashland, MA, USA). This colorimetric assay, first described by Mosmann (1983), is based on the ability of a mitochondrial dehydrogenase enzyme from viable cells to cleave tetrazolium rings of the pale yellow MTT and form dark blue formazan crystals. Approximately 1 h before the end of each loading experiment a MTT solution was prepared by dissolving 1 mg MTT per ml of the provided MTT diluent. A volume of 300 μl of MTT solution was added to all test wells containing EpiDerm cultures, and the plates were incubated at 37°C and 5% CO_2 for a period of 3 hours. After incubation, the cultures were removed from the MTT plates and gently blotted with paper tissue, before completely submerging them in 2 ml of the provided extractant solution per well. The extraction plates were placed in sealed bags to reduce evaporation and were gently shaken overnight in the dark at room temperature to extract the reduced MTT. For all samples, the extractant solution was pipetted up and down to ensure complete mixing, and finally, 200 μl were transferred to a 96-well plate for measuring the optical density (OD) using a plate reader set to 570 nm. In this study, the mean OD of the samples of the control group (C) was set to represent 100% viability.

IL-1α measurement

The levels of IL-1α were determined in the culture supernatant of all EpiDerm cultures by ELISA (Quantikine, R&D systems, Uithoorn, NL), with a detection limit of 1 pg/ml. Briefly, 200 μl culture supernatant or test standard were pipetted into the appropriate pre-coated wells and incubated for 2 h at room temperature. After aspirating and washing each well, 200 μl of IL-1α conjugate was added, and the plates were incubated at room temperature for an additional 1 h. When repeating the aspirating and washing step, 200 μl of a tetramethylbenzidine/hydrogen peroxide (1:1 v/v) mixture was added to each well. Following incubation for 20 minutes at room temperature, during which the plate was protected from light, 50 μl of 2 N sulfuric acid was added to each well to stop the reaction. The OD was determined within 30 minutes, using a microplate reader set to 450 nm. To correct for optical imperfections in the well plate, wavelength correction was performed at 570 nm.

2.2.4 Statistics

The results of the different experimental groups (C, PC, LP, and HP group) are expressed as mean $\pm$ standard error of the mean (SEM). Analyses of Variance (ANOVA) (STATGRAPHICS Plus 5.1, Statistical Graphics Corporation, USA) were performed to test differences in IL-1α release between the experimental groups of each loading experiment, as well as between the corresponding loading experiments (inter-batch differences) (table 2.1). For these analyses the IL-1α data was log transformed to normalize their distribution. Where ANOVA yielded significance, post hoc analyses were performed using Tukey's multiple comparisons test. Differences were considered to be significant at a P-value$<$0.05.

2.3 Results

A summary of the response of the epidermal equivalents to the different loading regimes is given in table 2.2.

2.3.1 Histological examination

Tissue damage was qualitatively assessed by histological examination of the region that was in direct contact with the polycarbonate plate or indenter, referred to as the loading region. The presence of a plate on the top surface of the EpiDerm cultures, for either a short- or long-term period, had no visible effect on the tissue structure and cytoplasmic RNA content (C and PC group, figure 2.2a-d and table 2.2). Furthermore, both low- and high-pressures (i.e. LP and HP group) did not seem to induce any visible tissue damage in the short-term loading experiments (table 2.2). In the long-term loading experiments, however, some swelling of the stratum spinosum and a small decrease in cytoplasmic RNA were observed in the LP group (figure 2.2e-f and table 2.2). At the same time, severe epidermal changes were observed in the HP group

	Batch 1	Batch 2	Batch 3	Batch 4	
Tissue damage	-	-	-	-	**C**
	-	-	-	-	**PC**
	-	-	+	+	**LP**
	-	-	++	++	**HP**
Viability [%]	NA	NA	100±2	100±0	**C**
	NA	NA	94±1	100±1	**PC**
	NA	NA	90±0	96±3	**LP**
	NA	NA	85±6	96±0	**HP**
IL-1α [pg/ml]	14±2	22±4	58±1	24±1	**C**
	18±2	22±6	38±14	16±2	**PC**
	50±9	37±7	161±39	49±16	**LP**
	77±4	75±35	484±140	258±68	**HP**

Table 2.2: Damage assessment in the different experimental groups (C, PC, LP, and HP groups) after both the short- (batch 1-2) and long-term (batch 3-4) loading experiments (mean±SEM). The viability results are normalized to the C group. The + and − symbol indicate structural tissue damage. NA indicates data not available.

(figure 2.2g-h and table 2.2). Increased cell swelling and a decrease in cytoplasmic RNA were observed, as well as some evidence of necrosis. The stratum corneum of these EpiDerm cultures appeared swollen, and the structure of this layer appeared poorly attached when compared to the C and PC group. Furthermore, the other epidermal layers (i.e. stratum basale, stratum spinosum, stratum granulosum) could no longer be distinguished from one another. In the tissue adjacent to the loading region no damage was observed (data not shown).

2.3.2 Viability measurement

The tissue viability was quantitatively assessed by a MTT assay and gives an indication of the amount of tissue damage. In contrast with the obtained histological results, the viability results imply that all EpiDerm cultures were resistant to prolonged mechanical loading (table 2.2). Indeed, only very small decreases in viability were found in both pressure groups (LP: 90-96% viable, HP: 85-96% viable) after the long-term loading period. Furthermore, the presence of the small plate on top of the EpiDerm cultures did not seem to affect the epidermal viability of batch 4, and only minimally affected viability (94%) in the PC group of batch 3.

2.3.3 IL-1α measurement

The release of IL-1α was assessed by a quantitative sandwich enzyme immunoassay technique, in order to detect the damaging effects of mechanical loading at an early

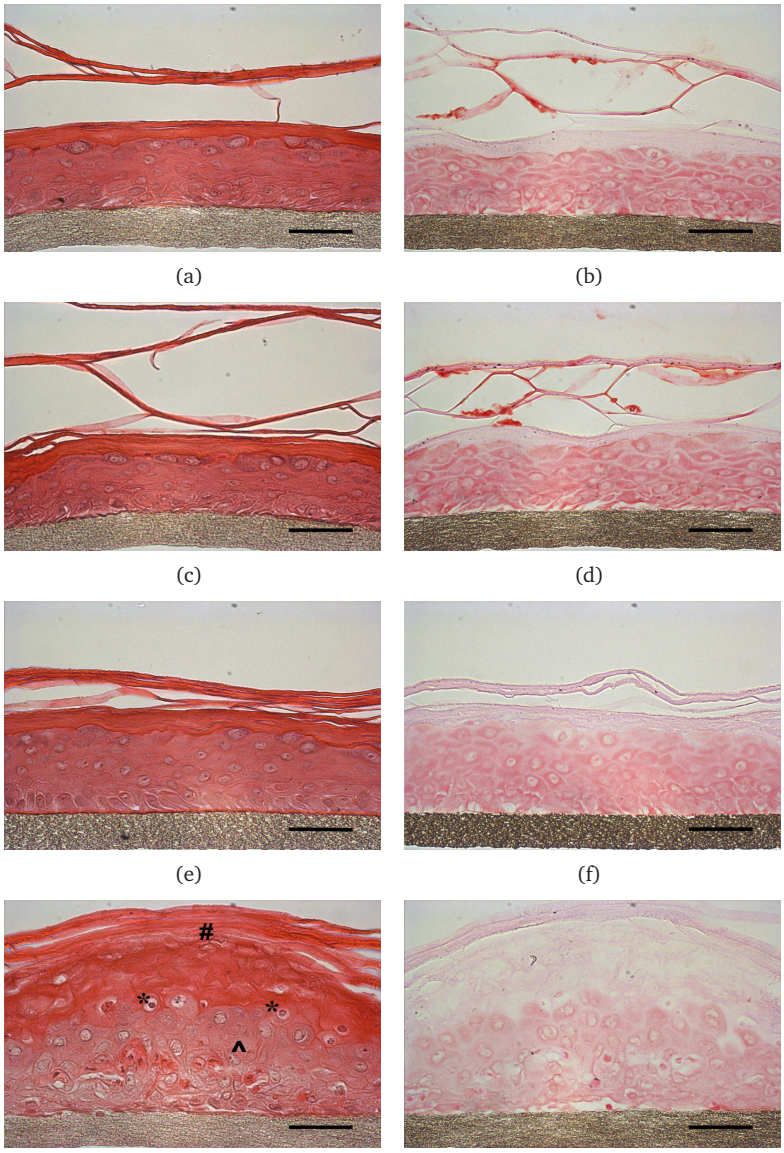

Figure 2.2: Epidermal histology after the long-term loading period: (a, b) the control (C), (c, d) the plate control (PC), (e, f) the low-pressure (LP), and (g, h) the high-pressure (HP) group. The short-term loading experiments are comparable to the displayed C and PC group (a-d). H&E (a, c, e, and g) and Pyronine-Y (b, d, f, and h) staining are shown. $*$ indicates cellular necrosis, $\wedge$ indicates cell swelling, and # indicates disruption of stratum corneum. The bars display a distance of 50 μm.

stage. The absolute values of IL-1α release, expressed in pg/ml, are shown in table 2.2. In figure 2.3 the IL-1α release of the different EpiDerm batches, expressed as percentage of the C group, are shown for both the short- and long-term loading experiments. After the short-term loading period an increase in IL-1α release was observed in both the LP and HP groups (figure 2.3a). This effect was most pronounced in the HP groups in which a 3.4-5.3 fold increase was observed in comparison with the C groups (batch 1: 5.3-fold increase, $P<0.005$). The presence of the small plate on top of the EpiDerm cultures did not seem to induce epidermal damage, since no significant increase in IL-1α release was observed in the PC groups. After the long-term loading period the same trend in IL-1α release was found (figure 2.3b). However, an even more pronounced increase in IL-1α release was observed in the HP groups of the long-term loading experiments as compared to the short-term loading experiments. The release of IL-1α of the HP groups increased by 8.3-fold (batch 3) and 10.9-fold (batch 4) when compared to the corresponding C groups (in both cases $P<0.005$). Furthermore, a significant difference was found in the IL-1α secretion in the culture supernatant between the HP and LP groups of batch 4 ($P<0.005$). The responses of the LP groups and the PC groups were comparable for both the short- and the long-term loading experiments.

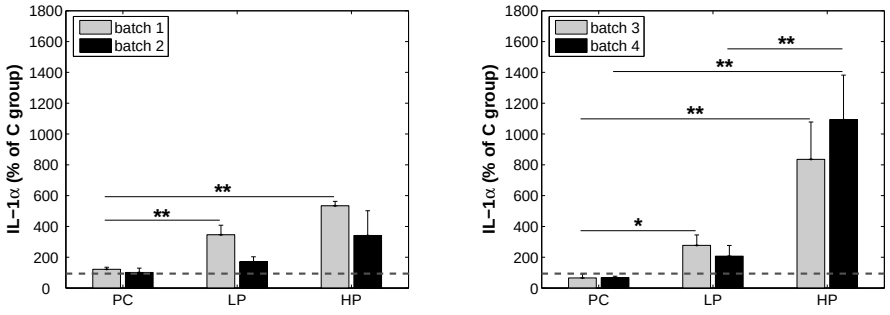

Figure 2.3: IL-1α release (mean+SEM) of the plate control (PC) group, the low-pressure (LP) group, and the high-pressure (HP) group, expressed as percentage of the control (C) group (dashed line), after both a short- (a) and long-term loading period (b). * represents $P<0.05$ and ** represents $P<0.005$

2.3.4 Batch variation

The suitability of the custom-built loading device to apply pressure-induced epidermal damage could be estimated from intra-batch differences in tissue viability and IL-1α release (i.e. data scatter within the different experimental groups) (table 2.2). Only

small differences in tissue viability were detected within the experimental groups of each loading experiment (i.e. each batch of EpiDerm cultures). Whereas, the intra-batch differences in IL-1α release seemed to increase with both magnitude and duration of epidermal loading (table 2.2). A significant relationship was found between the mean release of IL-1α and the standard error of the mean (SEM) (R^2=0.96, P<0.01, data not shown). On the other hand, inter-batch differences (i.e. difference between corresponding loading experiments) give an indication of the biological variation among different batches of EpiDerm cultures (table 2.2). The IL-1α release of batches 3 and 4 significantly differed from one another. These batches not only responded differently to prolonged loading, but also displayed a different basal release of IL-1α in their C groups (58$\pm$1 versus 24$\pm$1 pg/ml; table 2.2).

2.4 Discussion

Prolonged mechanical loading of soft tissues, such as skin, can eventually lead to tissue breakdown in the form of pressure ulcers. Existing histopathological data suggest a cellular origin to pressure ulcer development, therefore recent studies on soft tissue focus on the role of cell deformation in this damage process (Bouten *et al.*, 2001, 2003b; Berg and Rudolph, 1995). Due to the functional anaerobic nature of the epidermis, the avascular layer of the skin, sustained cell deformation might also underlie pressure ulcer formation in this tissue layer (Ronquist *et al.*, 2003). Because it is impossible to study cellular responses to loading *in vivo* independent of other factors, an *in vitro* model of the epidermis (i.e. EpiDerm) under mechanical loading was used in the present study. Different batches of EpiDerm cultures were subjected to 6.7 kPa (50 mmHg) (low-pressure: LP) and 13.3 kPa (100 mmHg) (high-pressure: HP) for either a short- or long-term loading period using a custom-built loading device (figure 2.1 and table 2.1). Unloaded cultures (control: C) and cultures loaded with a small plate of negligible weight (plate control: PC) were used as controls in this study. The damaging effects of prolonged mechanical loading were determined by various endpoint measurements. First, tissue damage was assessed by histological examination. Second, the viability of the epidermal equivalents was determined. And third, the release of a pro-inflammatory mediator, IL-1α, was measured as a marker of the onset of tissue damage.

No histological changes were observed in the tissue structure of the four different experimental groups after the short-term loading period (table 2.2). However, after the long-term loading period, some mild epidermal damage was observed in the LP groups (figure 2.2e-f and table 2.2). Some cell swelling was found in the stratum spinosum, as well as a small decrease in cytoplasmic RNA. Cell swelling or cytoplasmic swelling is known to be one of the first histological signs of cell damage (Cobb *et al.*, 1996). The decreased cytoplasmic RNA also indicates initial cell damage, which can eventually lead to necrosis (Jacobs *et al.*, 2000, 2002). Severe epidermal damage was only observed in the HP groups (figure 2.2g-h and table 2.2). Increased cell swelling, loss of distinguishable epidermal layers, and a decrease in cytoplasmic RNA were found, as well as some evidence of cell necrosis. Furthermore, the stratum

corneum of these EpiDerm cultures seemed swollen, and relatively detached when compared with the C and PC groups. These results indicate that the keratinocytes are not only damaged, but that the barrier function of the EpiDerm cultures might also be affected as a result of sustained loading. Immunohistochemistry might, further, improve insight in the damage development of the various epidermal layers upon sustained mechanical loading of the *in vitro* model system.

In contrast with the obtained histological results, only very small decreases in viability were observed after the long-term loading experiments (table 2.2). These viability results might, however, not give a perfect description of the actual tissue conditions due to limitations of the MTT viability assay. This assay is based on the reduction capacity of dymethylthiazol diphenyltetrazolium bromide (MTT) in the mitochondria of metabolically active cells (Faller and Bracher, 2002). Damage to the stratum corneum cannot be determined with this assay, since the corneocytes are no longer metabolically active. Furthermore, the three-dimensional structure of the epidermis might affect the penetration of the used MTT solution and, thereby, influence the sensitivity and specificity of the viability assay (Jacobs *et al.*, 2002). The penetration of MTT might even further be impeded under tissue loading. Keratinocyte damage, without disruption of cell membrane, might also not be detected using this method (Augustin and Damour, 1995; Jacobs *et al.*, 2002). In spite of these limitations, the obtained viability results give an indication of keratinocyte breakdown (i.e. necrosis) as a result of sustained epidermal loading. Other 'damage markers', should always be used in conjunction with the MTT viability assay to get a more complete description of the tissue conditions.

The exact role of pro-inflammatory cytokines, such as IL-1α in the development of skin ulcers is not known. It is, however, hypothesized that IL-1α is one of the first markers that is released upon the onset of skin ulcers. Previous research demonstrated that Il-1α is an important inflammatory mediator in the skin, and is believed to form the main switch in the induction of an inflammatory cascade (Coquette *et al.*, 2003; Kupper, 1990; Luger, 1989; Welss *et al.*, 2004). Cytokines are, furthermore, known to play a distinct role in the various phases of wound healing (Bronneberg and Bouten, 2005). In chronic wounds, such as pressure ulcers in an advanced stage, the normal wound healing process is impaired and the ulcers are locked in a state of chronic inflammation (Diegelmann and Evans, 2004). Indeed it has been postulated that this might be due, among other things, to abnormal levels of pro-inflammatory cytokines in the wound bed (Mast and Schultz, 1996).

IL-1α is constitutively expressed in keratinocytes and accumulates in the keratinocytes of all epidermal layers, mostly in the cytoplasm (Dinarello, 1998; Uchi *et al.*, 2000; Welss *et al.*, 2004). In an intact epidermis, the IL-1α reservoir is naturally eliminated by desquamation, due to the fact that IL-1α has no hydrophobic leader sequence for transmembrane secretion (Welss *et al.*, 2004). Therefore, IL-1α is only released from leaky cells following damage (Dinarello, 1998; Welss *et al.*, 2004). Currently, IL-1α is widely accepted for screening the damaging potential of chemical irritants before the physiological signs of skin irritation occur (Corsini *et al.*, 1996; Corsini and Galli, 1998; Faller and Bracher, 2002; Gibbs *et al.*, 2002). The present study used IL-1α as a marker to determine the effects of prolonged mechanical load-

ing of the epidermis before the onset of visible tissue breakdown. After the short-term loading period an increase in IL-1α release was observed that was the most pronounced in the HP groups (table 2.2 and figure 2.3a). No histological changes were, however, observed in the different experimental groups after this loading period (table 2.2). Therefore, these results indicate that short-term epidermal loading caused some cell damage that cannot be demonstrated by standard microscopic techniques. After the long-term loading period the same trend in IL-1α release was found (table 2.2 and figure 2.3b). However, an even more severe increase in IL-1α release was observed in the HP groups of the long-term loading experiment as compared to the short-term loading experiment, which corresponds well to the observed epidermal damage and decrease in tissue viability (table 2.2 and figure 2.2g-h). Furthermore, the HP and LP groups of batch 4 were significantly different from one another, which indicates that IL-1α is capable to distinguish between the two applied pressure levels. The various end-point measurements indicate that the application of a small plate on top of the EpiDerm cultures did seem to induce significant epidermal damage. Therefore, this experimental group will be omitted from future experiments.

In the present study, intra- and inter-batch differences in tissue viability and IL-1α release are observed, that are comparable to studies on cutaneous irritancy testing (table 2.2) (Faller and Bracher, 2002). Therefore, the loading device seems suitable to apply pressure-induced epidermal damage. However, the sample size of the experimental groups should be increased in future experiments to account for increasing intra-batch differences with increased magnitude and duration of epidermal loading. The observed inter-batch differences in IL-1α were generally higher than the intra-batch differences and are probably caused by biological variance. In future experiments, various batches of EpiDerm cultures should be analyzed to account for these differences.

The selected *in vitro* model system can be used to examine the effects of cell deformation, during prolonged mechanical loading of epidermal equivalents, independently of other factors (such as blood perfusion) that are known to be implicated in the development of pressure ulcers. Such a system enables improved control of experimental conditions, and offers the potential of performing well-controlled loading experiments. However, care should be taken when extrapolating the obtained *in vitro* results to a clinical setting. For instance, the mechanical properties of the *in vitro* model of the epidermis, and hence epidermal deformation, may differ significantly from those *in vivo*, the latter of which includes consideration of both pre-strain and inherent material properties. A computational model should provide more insight in the local deformations of the epidermis during sustained mechanical loading and is currently under development in our group.

In conclusion, we have described a new model system to study the damaging effects of prolonged mechanical loading in the epidermis under *in vitro* conditions. This model system is easy to use and is suitable to apply sustained pressures to epidermal equivalents. Various animal models, looking at ulcer formation in the skin, demonstrated that shear stresses also caused superficial skin damage (i.e. detachment of the stratum corneum), and may reduce the time to develop visible tissue damage in combination with pressure (Dinsdale, 1973, 1974; Goldstein and Sanders, 1998).

Therefore, future studies should focus on the damaging effects of these stresses. Various loading regimes (i.e. loading modes (pressure and/or shear stress), magnitudes, and durations) might be imposed on the described *in vitro* model of the epidermis to obtain an indication of the critical loading-time relationship for superficial skin ulcer formation. The present study demonstrated that the analyses of various end-point measurements, such as tissue histology, tissue viability, and IL-1α release, increase the understanding of the epidermal damage process due to prolonged mechanical loading. Therefore, future studies will proceed using these end-point measurements to broaden the understanding of the aetiology and pathogenesis of superficial skin ulcers. The detection of a damage marker, such as IL-1α, was shown to be of particular importance, since epidermal damage could be detected before the onset of visible tissue breakdown. IL-1α might, therefore, be a promising marker for detecting pressure ulcers *in vivo* at an early stage. In future experiments various damage markers will be studied *in vitro* as well as *in vivo* and will be tested for their role in pressure ulcer prevention.

2.5 Acknowledgements

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Chapter 3

Cytokine and chemokine release upon mechanical loading of the epidermis

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3.1 Introduction

Pressure ulcers are areas of soft tissue breakdown that result from sustained mechanical loading of the skin and underlying tissues (APUAP, 1998). In the skin these ulcers are presented by the following sequence of stages: blanchable hyperemia, non-blanchable hyperemia, ecchymosis (haemorrhagic spot) and finally tissue necrosis (Peirce *et al.*, 2000). Pressure ulcers are painful, difficult to treat, and represent a burden to the community in terms of health care and money (Bouten *et al.*, 2003b). The prevalence of pressure ulcers is unacceptably high and have been reported to vary from 13% in hospitalized patients to 35% in institutions for the physically handicapped (Bours *et al.*, 2002). In our view, this high prevalence is partly due to limited risk assessment techniques. Currently, risk assessment is mainly performed by questionnaires or scales like the Norton (Norton, 1989), Braden (Bergstrom *et al.*, 1987), Waterlow (Waterlow, 1985), and CBO (CBO, 2002). Most risk assessment scales are based on expert opinion or literature review and have a limited scientific background (Schoonhoven *et al.*, 2002). Schoonhoven *et al.* (2002) demonstrated that these risk assessment scales do not predict the degree of susceptibility with the required accuracy, since up to 30% of the patients with pressure ulcers are still misclassified. Therefore, objective measures are needed for identifying patients 'at risk' of developing pressure ulcers. Evaluating markers (proteins) for early detection of these ulcers is an essential step in developing an improved risk assessment method.

The study in chapter 2 showed that sustained mechanical loading of the epidermis, the superficial layer of the skin, increased the release of interleukin 1 α (IL-1α) in an *in vitro* model of pressure ulcer development (Bronneberg *et al.*, 2006). IL-1α is a pluripotent, multifunctional cytokine that plays a key role in the initiation and development of inflammatory responses by inducing the production of various chemokines (Uchi *et al.*, 2000). This finding led to the hypothesis that superficial pressure ulcers are preceded by an inflammatory response. Cytokines and chemokines (IL-1α, interleukin 1 receptor antagonist (IL-1RA), interleukin 8 (IL-8), tumor necrosis factor α (TNF-α), macrophage inflammatory protein 3 α (MIP-3α), monocyte chemotactic protein 1 (MCP-1), and growth-regulated protein precursor α (GRO-α)), which originate from keratinocytes in the epidermis (Uchi *et al.*, 2000), might therefore be suitable markers for early detection of superficial pressure ulcers.

Mechanical straining of keratinocyte monolayers is known to promote the release of IL-1α and IL-1RA (Lee *et al.*, 1997). In keratinocytes, IL-1α is mostly stored in the cytoplasm and is released from leaky cells following damage (Dinarello, 1998; Uchi *et al.*, 2000; Wood *et al.*, 1996). At this moment IL-1α is widely used for screening the damaging potential of chemical irritants before the physiological signs of skin irritation occur (Corsini *et al.*, 1996; Corsini and Galli, 1998; Faller and Bracher, 2002; Gibbs *et al.*, 2002). IL-1RA is a competitive inhibitor of IL-1α (Uchi *et al.*, 2000). Like IL-1α, TNF-α is also suggested to be released as an inflammatory mediator which triggers chemokine signals in response to danger from the environment (Spiekstra *et al.*, 2005; Kock *et al.*, 1990). Chemokines, such as IL-8, MIP-3α, MCP-1, and GRO-α, are known to further mediate inflammatory responses (Spiekstra *et al.*, 2005; Uchi *et al.*, 2000).

The current study investigates whether cytokines and chemokines (IL-1α, IL-1RA, TNF-α, IL-8, MIP-3α, MCP-1, and GRO-α) are released as a result of sustained mechanical loading of the epidermis *in vitro*. For this purpose, commercially available epidermal equivalents (EpiDerm), of which the general morphology is comparable to human epidermis (Netzlaff *et al.*, 2005), are subjected to clinically relevant pressures (0, 50, 75, 100, 150, and 200 mmHg) (Kanj *et al.*, 1998; Wang and Sanders., 2005) for 24 h. These interface pressures can be experienced at the most common locations for pressure ulcers while sitting or lying in bed (sacrum: 62-107 mmHg, trochanter: 6-156 mmHg, heels: 107-213, and ischial tuberosities: 60-146 mmHg) (Swain, 2005). Increasing pressures are applied to the surface of the EpiDerm cultures to cause various degrees of epidermal damage. Cytokines and chemokines that are released when structural tissue damage is barely visible, are of interest in this study and might be suitable for early detection of pressure ulcers *in vivo*.

3.2 Materials and methods

3.2.1 Epidermal equivalent

A commercially available human epidermal equivalent, EpiDerm (EPI-200, MatTek Corporation, Ashland, MA, USA), was used as an *in vitro* model of the epidermis in this study (Cannon *et al.*, 1994). These EpiDerm cultures (diameter=8 mm) are comprised of human-derived epidermal keratinocytes, which have been cultured on standing cell culture inserts (Millipore, Billerica, MA, USA) at the air-liquid interface to form a multilayered, differentiated model of the human epidermis. The data sheet from the MatTek Corporation further indicates that these cell culture inserts consist of porous, flexible culture membranes of polycarbonate, which have been chemically modified and collagen coated.

Upon receipt, the EpiDerm cultures were placed in 6-well plates and were cultured overnight at 37°C and 5% CO_2 in 900 μl/well of hydrocortisone free maintenance medium (EPI-100-MM-HCF, MatTek Corporation, Ashland, MA, USA). The next morning, the cultures were transferred to fresh medium and directly used in a loading experiment. Four separate batches of EpiDerm cultures (n=24) were used.

3.2.2 Pressure application

Pressures of 0, 50, 75, 100, 150, and 200 mmHg were applied to the top surface of the EpiDerm cultures for a period of 24 h, using a custom-built loading device (Bronneberg *et al.*, 2006). Indenters (diameter=5 mm) and additional weights were used for applying specific pressures on top of the cultures (figure 3.1). After the loading experiments, the culture supernatants were collected and stored at -80°C for subsequent analysis of extracellular cytokines and chemokines, as well as analysis of cell membrane integrity. The EpiDerm cultures were washed twice with the provided phosphate buffered saline (PBS, MatTek Corporation, Ashland, MA, USA) and were formalin fixed for histological examination.

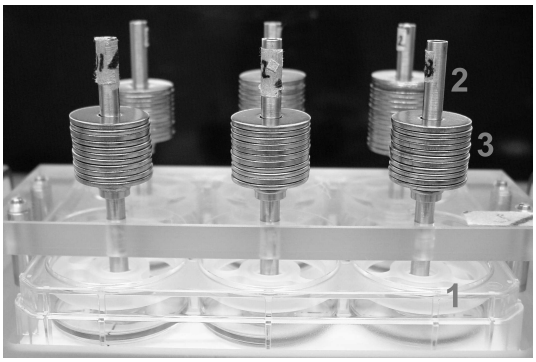


Figure 3.1: Custom-built loading device used to apply sustained pressures on six EpiDerm cultures (1) simultaneously. Pressures were applied using indenters (2) and additional weights (3).

3.2.3 Damage assessment

Histological examination

The EpiDerm cultures were fixed in 10% phosphate-buffered formalin and processed for conventional paraffin embedding. 5 μm sections were cut and stained with haematoxylin and eosin (H&E). Tissue morphology was studied by light microscopy.

Membrane integrity

The cell membrane integrity (cell damage) of the EpiDerm cultures was measured by a colorimetric lactate dehydrogenase (LDH) assay (TOX-7, Sigma-Aldrich Co, St. Louis, Missouri, USA) according to recommendations from the supplier. This assay measures the membrane integrity as function of the amount of cytoplasmic LDH released into the culture supernatant (Legrand *et al.*, 1992). Briefly, the assay mixture was prepared by mixing equal amounts of LDH assay substrate, cofactor and dye solutions. For all cultures, assay mixture was added to the culture supernatant in a proportion of two to one. After incubation for 30 min at room temperature in the dark, the color reaction was stopped by 1 M HCL. Absorbance was determined at 490 nm using a plate reader. Background correction was performed at 650 nm.

3.2.4 Cytokine and chemokine release

The levels of IL-1α, IL-1RA, TNF-α, IL-8, MIP-3α, GRO-α, and MCP-1 were determined in the culture supernatants by ELISA. TNF-α was quantified by a Quantikine high sensitive kit (HSTA00C, R&D systems, Abingdon, United Kingdom) according to the recommendations from the supplier. For IL-1α, IL-1RA, IL8 (M9318, CLB, Amsterdam, the Netherlands), MIP-3α, GRO-α, and MCP-1 quantification, immunoplates (Nalge

Nunc International, Roskilde, Denmark) were coated overnight at room temperature with capture antibody. Subsequently, the plates were blocked with PBS/0.5% BSA for 1 h at room temperature, after which the culture supernatants and standards were added to the plate. After incubation for 1 h at room temperature and washing (PBS/ 0.005% Tween-20), the detection antibody was added. The plates were subsequently incubated with 1:10000 diluted streptavidin-HRP (M2032, Sanquin Reagents, Amsterdam, The Netherlands) for a period of 30 min. The enzyme reaction was initiated by adding 0.2 mg/ml of o-phenylenediamine dihydrochloride in 0.11 M acetate pH 5.5 and 0.03% hydrogen peroxide and stopped with 2M H_2SO_4. Absorbance was determined at 490 nm using a plate reader. All antibodies and standards were purchased from R&D systems (Abingdon, United Kingdom), unless otherwise indicated.

3.2.5 Statistics

The amount of LDH as well as the amount of cytokines and chemokines released by the EpiDerm culture are expressed as mean + standard error of the mean (SEM) per level of applied pressure. Analyses of Variance (ANOVA) with a randomized block design (SPSS 12.0.1, Chicago, Illinois, USA), followed by a Dunnet's multiple comparison test, was used to compare the release at each pressure level (50, 75, 100, 150, and 200 mmHg) to the unloaded control group (0 mmHg). Differences were considered to be significant at a P-value$<$0.05.

3.3 Results

3.3.1 Damage assessment

An increasing degree of epidermal damage was observed with increasing pressures applied on the top surface of the EpiDerm cultures. This damage was characterized by deleterious changes in the tissue architecture, for example, loosening of the stratum corneum, loss of distinguishable epidermal layers, increased number of vacuoles, cell swelling, and necrosis and by an increase in extracellular LDH level (figure 3.2 and 3.3). At 50 and 75 mmHg, minor tissue damage was observed compared to the unloaded control group (0 mmHg) (figure 3.2a-c). The stratum granulosum could no longer be detected at these pressures. Furthermore, an increase in LDH was observed at pressures up to 75 mmHg, although not significantly different from the unloaded control group (figure 3.3). However at 100 mmHg, tissue damage was further enhanced. The stratum corneum was less compact and swollen cells, vacuoles, and necrosis appeared throughout the complete upper part of the epidermis (figure 3.2d). In addition, the different layers of the epidermis could no longer be distinguished at this pressure. Furthermore, a significant increase in the release of LDH was observed compared to the control group (8.4-fold, P$<$0.05) (figure 3.3). The most severe tissue breakdown was, however, found after loading the EpiDerm cultures with 150 and 200 mmHg (figure 3.2e-f). In addition to the aforementioned structural tissue damage, the lower part of the epidermis appeared completely compressed. Furthermore, an

even higher increase in LDH was measured at these pressures compared to the control group (150 mmHg: 10-fold, 200 mmHg: 12-fold, $P<0.01$) (figure 3.3).

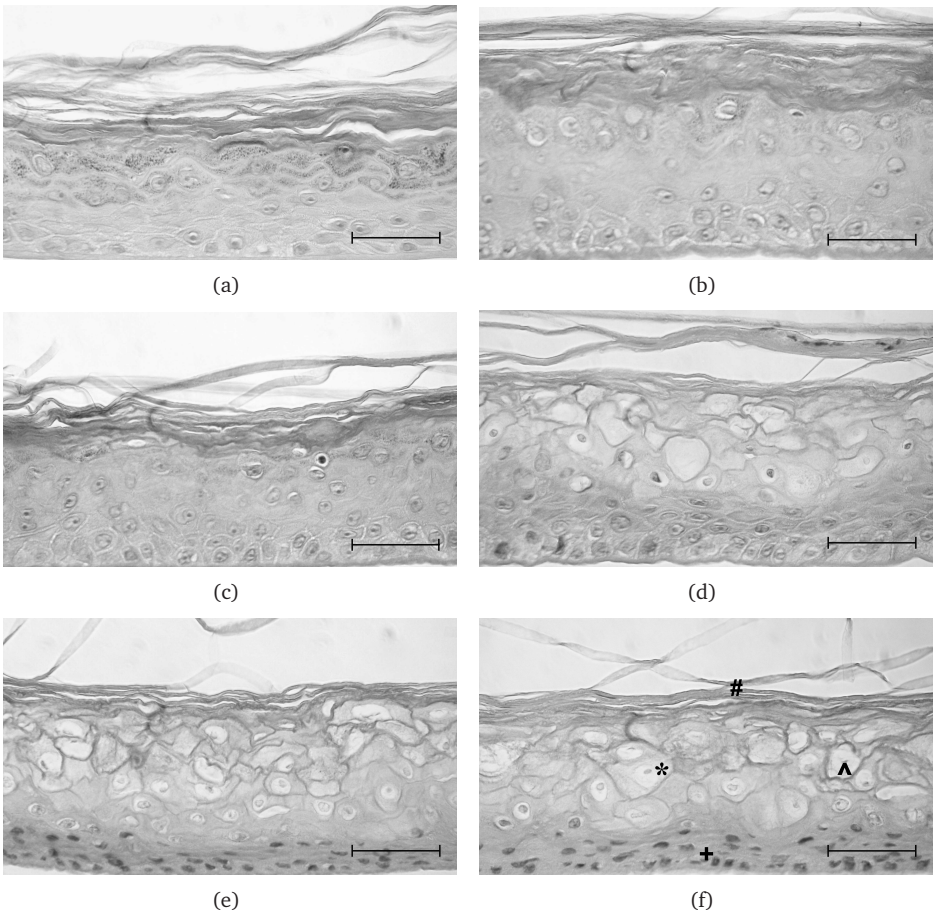


Figure 3.2: Epidermal histology of the different experimental groups after 24 h of loading: 0 mmHg (a), 50 mmHg (b), 75 mmHg (c), 100 mmHg (d), 150 mmHg (e), and 200 mmHg (f). Images were obtained in the middle of the loading region. $*$ indicates cellular necrosis, $\wedge$ indicates cell swelling, # indicates disruption of stratum corneum, and + indicates compressed cells. The bars indicate a distance of 50 μm.

In summary, three different degrees of tissue damage could be distinguished: minor (50 and 75 mmHg), moderate (100 mmHg), and severe (150 and 200 mmHg) epidermal damage.

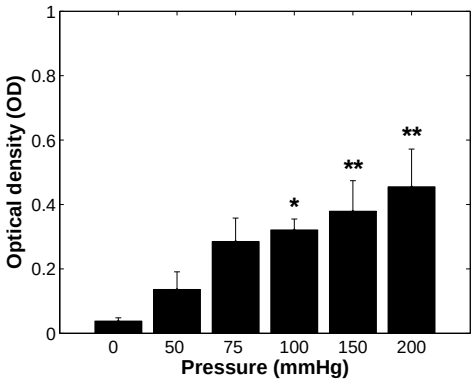


Figure 3.3: LDH release (mean+SEM), expressed as optical density (OD), of the different experimental groups after 24h of loading. * indicates P$<$0.05 and ** indicates P$<$0.01 compared to the unloaded control group (0 mmHg).

3.3.2 Cytokine and chemokine release

Various cytokines and chemokines (e.g. IL-1α, IL-1RA, IL-8, and TNF-α) were measured upon sustained epidermal loading (figure 3.4). The release of IL-1α, IL-1RA, and IL-8 followed a distinct step pattern (figure 3.4a-c). Only small changes occurred at 50 mmHg, whereas, at 75 mmHg there were significant increases compared to the control group of IL-1α (4.8-fold, P$<$0.01), IL-1RA (5.7-fold, P$<$0.001), and IL-8 (2.9-fold, P$<$0.05). With pressures beyond 75 mmHg, the levels of the cytokines and chemokines remained fairly constant. This suggested an identification of a specific threshold for the release of cytokines and chemokines above which a strong increase could be detected. By contrast TNF-α increased monotonically with the applied pressures, the difference being statistically significant at 100 mmHg and beyond (figure 3.4d). No significant increase in the levels of MIP-3α and GRO-α were observed (data not shown), whereas MCP-1 could not be detected using the current analyses method.

3.3.3 Batch variability

Different batches of EpiDerm cultures were used in the current study. In figure 3.5 the release patterns of IL-1α and TNF-α are shown for the different batches of EpiDerm cultures. From this figure it can be concluded that the individual batches respond somewhat different to sustained mechanical loading. For IL-1α, as well as IL-1RA and IL-8 (data not shown), high inter-batch differences were observed at pressures of 75, 100, 150, and 200 mmHg (figure 3.5a). Whereas for TNF-α less inter-batch differences could be detected (figure 3.5b).

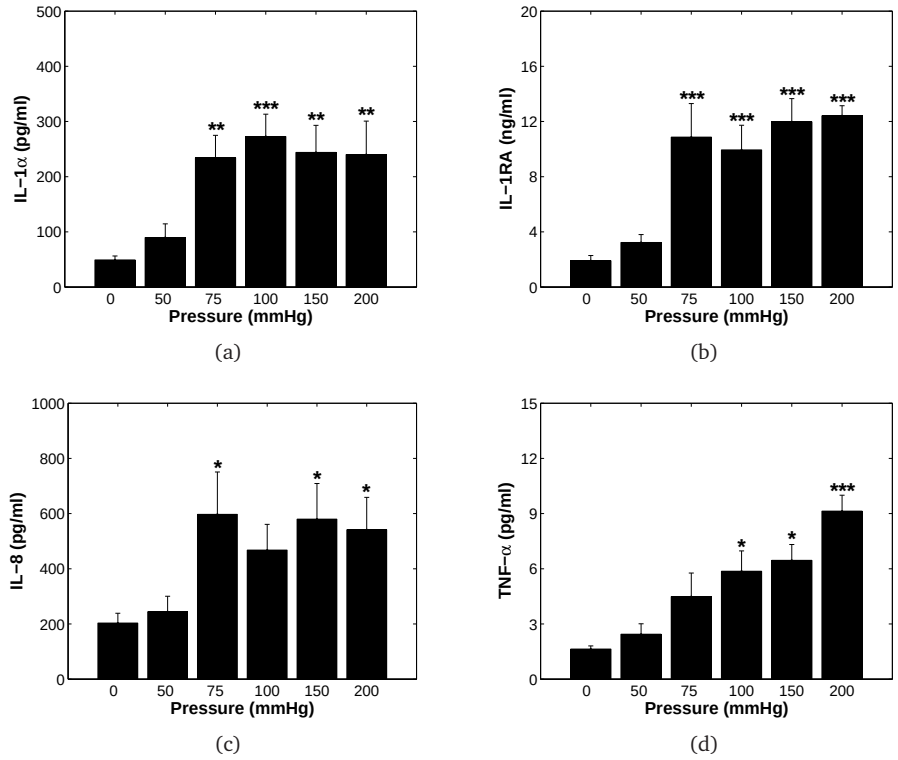
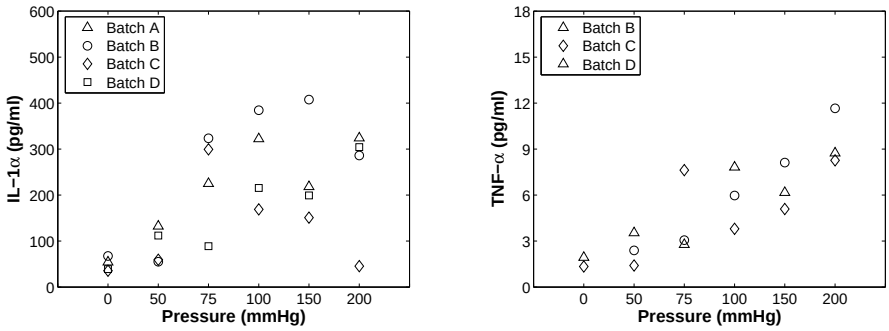


Figure 3.4: IL-1α (a), IL-1RA (b), IL-8 (c), and TNF-α (d) release (mean+SEM) of the different experimental groups after 24h of mechanical loading. * indicates P$<$0.05, ** indicates P$<$0.01, and *** indicates P$<$0.001 compared to the unloaded control group (0 mmHg).

3.4 Discussion

Cytokines and chemokines are known mediators of the inflammatory response, which is a tissue response to irritation and/or injury and is characterized by pain, swelling, redness, and heat. Goldstein and Sanders (1998) previously demonstrated that superficial pressure ulcers (stage I ulcers) in pigs showed morphological signs of inflammation. It is therefore hypothesized that pressure ulcers are preceded by an inflammatory response. In the present study we investigated the extracellular release of cytokines and chemokines (IL-1α, IL-1RA, TNF-α, IL-8, MIP-3α, MCP-1, and GRO-α) upon sustained mechanical loading of the epidermis *in vitro*. Increasing pressures (0, 50, 75, 100, 150, and 200 mmHg) were applied to irritate and/or injure epidermal tissue and cause various degrees of tissue damage. Cytokines and chemokines that are released *in vitro* when epidermal damage is barely visible, are of interest in



(a) (b)

Figure 3.5: IL-1α (a) and TNF-α (b) release for the individual batches of EpiDerm cultures after 24h of mechanical loading.

this study as they might prove to be suitable markers for early detection of superficial pressure ulcers *in vivo*.

The commercially available EpiDerm cultures were used in the present study. The general morphology of the EpiDerm cultures is comparable to that of normal human epidermis (Netzlaff *et al.*, 2005). The cells in the stratum basale appear columnar to round in morphology. In the stratum spinosum the cells are flattened, as in native epidermis. The lamellar bodies in the stratum granulosum appear normal, and the keratohyalin granules appear rounded to satellite in shape. Furthermore, it was shown that the stratum corneum of the EpiDerm cultures exhibit a normal basket-weave pattern and that the extrusion of the lamellar body contents at the interface between the stratum granulosum and the stratum corneum is complete (Ponec *et al.*, 2002). Lipid analysis, further, revealed the presence of all major lipid classes in the EpiDerm cultures (Ponec *et al.*, 2002). The expression and location of differentiation markers was also determined in these cultures (Ponec *et al.*, 2002; Netzlaff *et al.*, 2005). As in native epidermis, keratin 1 and 10 were expressed in all suprabasal layers of the EpiDerm cultures. Furthermore, loricrin and SPRR2 were located in the stratum granulosum. Involucrin (all suprabasal layers) and transglutaminase (upper suprabasal layers) were also expressed in the EpiDerm cultures, but their location slightly differed from that of native epidermis (stratum granulosum). Since, EpiDerm cultures have many characteristics of human epidermis, they provide a good model to study the effect of sustained mechanical loading *in vitro*. One of the main advantages of using an *in vitro* model system is that it enables studying the response to tissue deformation in a controlled manner, which is independent of other predisposing factors of pressure ulcers that are present *in vivo*, such as ischemia, impaired interstitial fluid flow and lymphatic drainage, and reperfusion (Bouten *et al.*, 2003b; Daniel *et al.*, 1981; Peirce *et al.*, 2000; Reddy and Cochran, 1981).

Three degrees of epidermal damage could be distinguished corresponding to in-

creasing levels of sustained pressure, namely minor, moderate, and severe tissue damage. The former, involves a poorly defined stratum granulosum which was observed after loading the EpiDerm cultures with 50 or 75 mmHg. At 100 mmHg, moderate tissue damage was found involving a less compact stratum corneum and swollen cells, vacuoles, and necrosis appearing throughout the complete upper part of the epidermis (i.e. stratum granulosum and stratum spinosum). These changes have functional consequences. For example a less compact stratum corneum might indicate loss of structure and function of the epidermal barrier. Cell swelling or cytoplasmic swelling is further known to be one of the first visible signs of cell damage (Cobb *et al.*, 1996). This cell damage is supported by the observed release in LDH (Decker and Lohmann-Matthes, 1998; Legrand *et al.*, 1992; Ponec and Kempenaar, 1995). Severe tissue damage was observed after loading the EpiDerm cultures with 150 and 200 mmHg. In addition to the earlier observed structural tissue damage, the lower part of the epidermis appeared completely compressed. This was accompanied by an even higher increase in LDH release. These observed results are comparable to earlier studies related to pressure ulcers. For example, a decrease in the mechanical properties (i.e. Youngs-modulus) of human skin tissue was reported on static loading with 170 mmHg, while no significant tissue changes were observed at 50 mmHg (Edsberg *et al.*, 1999). A decrease in mechanical properties arises from structural changes in the tissue architecture, as were observed in the current study.

Sustained mechanical loading of EpiDerm cultures resulted in an increase in the levels of IL-1α, IL-1RA, IL-8, and TNF-α. These results correspond well to studies performed by Lee *et al.* (1997) and Takei *et al.* (1998), which showed that mechanical straining of keratinocyte monolayers promotes the expression and release of IL-1α and IL-1RA. An increase in cytokines such as IL-1α and TNF-α was also found in studies in which epidermal tissue was irritated and/or injured by applying various chemicals, UV irradiation, or by tape stripping (Bernhofer *et al.*, 1999; Faller and Bracher, 2002; Flint *et al.*, 1998; Nickoloff and Naidu, 1994; Oxholm *et al.*, 1988; Wood *et al.*, 1992, 1997). Thus these cytokines seemed to be released as inflammatory mediators in response to any danger from the environment. Furthermore, it has been demonstrated that IL-1α and TNF-α stimulate the release of chemokines such as IL-8, MIP-3α, GRO-α, and MCP-1 in epidermal cultures (Nakayama *et al.*, 2001; Tohyama *et al.*, 2001; Steude *et al.*, 2002; Wetzler *et al.*, 2000). In the present study, an increase in IL-8 was observed, but no increase in the release of MIP-3α and GRO-α could be detected, and MCP-1 could not be measured at all. It is possible that other factors are involved in the mechanical damage process that attenuate the expression, synthesis, and release of these chemokines.

The release of IL-1α, IL-1RA, and IL-8 followed a distinct step pattern. A significant increase in the release of these cytokines and chemokines was observed at the relatively low pressure of 75 mmHg when tissue damage was minor. This finding indicates the sensitivity of the epidermis for these cytokines and chemokines at relatively low pressure levels. Nevertheless, IL-1α, IL-1RA, and IL-8 do not appear to discriminate between different degrees of tissue damage, since their levels remained nearly constant with increasing pressures. Furthermore, high inter-batch differences were observed, which are most probably due to factors such as, biological variation, cul-

ture conditions, and tissue handling during shipping. For TNF-α an increasing trend towards higher release levels was observed with increasing pressures. However, only relatively small levels of TNF-α were measured in the culture supernatant (i.e 4.5 pg/ml TNF-α versus 234.2 pg/ml IL-1α at 75 mmHg), which could only be detected using a high sensitive and expensive analysis technique.

At this moment, cytokines and chemokines are measured after a 24 h loading period, which is quite long and may in a clinical situation already lead to severe pressure ulcers. Therefore, it is an important next step to analyze the release of these cytokines and chemokines at intermediate time intervals during loading.

In conclusion, IL-1α, IL-1RA, IL-8, and TNF-α are released *in vitro* as a result of sustained mechanical loading. A first increase in the extracellular release of these cytokines and chemokines was observed at the relatively low pressure of 75 mmHg, when tissue damage was barely visible. In this respect, these cytokines and chemokines may prove to be suitable markers for the objective and early detection of pressure ulcer formation *in vivo*. A non-invasive method is, however, needed to assess these extracellular markers at the skin surface *in vivo* and is subject of current investigation. Furthermore, well-controlled *in vivo* studies need to be performed to verify the precise pressure-release profiles for the studied markers under these more complex conditions.

3.5 Acknowledgements

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Chapter 4

Early detection of mechanically-induced epidermal damage

The contents of this chapter are based on D. Bronneberg, L. H. Cornelissen, C. W. J. Oomens, F. P. T. Baaijens and C. V. C. Bouten (2007) *Early detection of mechanically-induced epidermal damage*, Experimental Dermatology, submitted.

4.1 Introduction

Pressure ulcers are areas of soft tissue breakdown, resulting from sustained mechanical loading of the skin and underlying tissues (APUAP, 1998). The prevalence of pressure ulcers is unacceptably high and have been reported to vary from 13% in hospitalized patients to 35% in institutions for the physically handicapped (Bours *et al.*, 2002). This high prevalence is partly due to limited risk assessment and detection techniques. Currently, risk assessment is mainly performed by questionnaires or scales such as the Norton (Norton, 1989), Braden (Bergstrom *et al.*, 1987), Waterlow (Waterlow, 1985), and CBO (CBO, 2002). Most risk assessment scales are based on expert opinion or literature review and have a limited scientific background (Schoonhoven *et al.*, 2002). Furthermore, Schoonhoven *et al.* (2002) demonstrated that risk assessment scales do not predict the degree of susceptibility with the required accuracy and indeed, up to 30% of the hospitalized patients with pressure ulcers are still misclassified. Accordingly, patients at high risk might not receive adequate preventive measures. The Dutch National Prevalence Survey demonstrated that grade I ulcers, the first stage of superficial ulcers in skin, accounted for approximately 50% of the prevalence of pressure ulcers (Bours *et al.*, 2002). Grade I ulcers are classified as non-blanchable erythema (NBE) of intact skin (Defloor *et al.*, 2005). Currently, the transparant disk method is commonly used for visual observation of NBE, in which the disk is pressed into the erythematous tissue. If the skin under this disk does not blanch, it is regarded as a grade I ulcer (Halfens *et al.*, 2001; Vanderwee *et al.*, 2006). For individuals with darker skin types, it is more difficult to determine grade I ulcers. Alternative measures, such as skin temperature, edema, induration or skin hardness might be used in these individuals. Nonetheless, Rosen *et al.* (2006) demonstrated that patients with darker skin types are still less likely to have their grade I ulcers identified compared to patients with lighter skin types. More importantly, the mentioned techniques aim for measuring the consequences of 'harm' already done to the skin (i.e. inflammation). Whereas pressure ulcer prevention should aim for earlier detection of developing ulcers. Improved pressure ulcer risk assessment is, therefore, necessary. A first step in developing a new risk assessment tool is directed towards the detection of skin reactions that precede grade I ulcers. Cytokines and chemokines are of interest for pressure ulcer detection, since they are known to mediate inflammatory responses. The present study focuses on cytokines and chemokines as biochemical markers for early detection of mechanically-induced epidermal damage.

Ideally, biochemical markers for pressure ulcer risk assessment should be measured at the skin surface in a non-invasive way. Therefore, cytokines and chemokines are evaluated that originate from cells in the superficial layer of the skin, the epidermis. Recently, interleukin 1 α (IL-1α), interleukin 1 receptor antagonist (IL-1RA), interleukin 8 (IL-8), and tumor necrosis factor α (TNF-α) have been identified as promising markers for pressure ulcer detection (chapter 3). Using an *in vitro* model system for pressure ulcer development, an increase in the release of these markers was observed after 24 h of loading with 0, 50, 75, 100, 150, and 200 mmHg. Commercially available EpiDerm cultures, of which the general morphology is comparable to human epidermis, were used as epidermal equivalents in these studies (Cannon

et al., 1994; Netzlaff *et al.*, 2005). Furthermore, Lee *et al.* (1997) and Takei *et al.* (1998), showed that mechanical straining of keratinocyte monolayers promotes the expression and release of IL-1α and IL-1RA.

Until now, the release of cytokines and chemokines (IL-1α, IL-1RA, IL-8, and TNF-α) was measured in the culture supernatant after a 24 h loading period, which is quite long and may, in a clinical situation, already lead to severe pressure ulcers. To be able to understand the regulation of cytokines and chemokines in the most superficial layer of the skin (i.e. the epidermis) in more detail and eventually extrapolate the results to a clinical setting, it is important to have an idea on the pressure-time release profiles and transport properties of these markers. Cytokines and chemokines are only suitable biochemical markers for early detection of pressure ulcers when they are quickly released upon mechanical loading. Thus, before the onset of structural tissue damage. A quick release might be possible when these cytokines and chemokines are stored intracellularly in the epidermis in a normal situation.

The aim of the present study is to evaluate the 'pressure-time' regulation of cytokines and chemokines (IL-1α, IL-1RA, IL-8, and TNF-α) in the epidermis *in vitro*. This study focusses on cytokines and chemokines that are released into the culture supernatant, as well as on the amount that remains inside the epidermal tissue. Epidermal equivalents are, therefore, subjected to either 0 or 150 mmHg in a custom-built loading device for various loading periods (1, 2, 4, 6, 8, 16, and 24 h).

4.2 Materials and methods

4.2.1 Engineered epidermal equivalent

A commercially available human epidermal equivalent, EpiDerm (EPI-200, MatTek Corporation, Ashland, MA, USA), was used as an *in vitro* model of the epidermis in this study (Cannon *et al.*, 1994). These EpiDerm cultures (diameter=8 mm) are comprised of human-derived epidermal keratinocytes, which have been cultured on standing cell culture inserts (Millipore, Billerica, MA, USA) at the air-liquid interface to form a multilayered, differentiated model of the human epidermis (Cannon *et al.*, 1994).

Upon receipt, the EpiDerm cultures were placed in 6-well plates, and were cultured overnight at 37°C and 5% CO_2 in 900 μl/well of hydrocortisone free maintenance medium (EPI-100-MM-HCF, MatTek Corporation, Ashland, MA, USA). The next day, the cultures were transferred to fresh medium and directly used in 'pressure-time' experiments. Seven batches of EpiDerm cultures, of twenty four cultures each, were used in this study.

4.2.2 Pressure-time experiment

A pressure of 150 mmHg was applied on the top surface of the EpiDerm cultures for various loading times (1, 2, 4, 6, 8, 16, and 24 h). This pressure was applied using indenters and additional weights (figure 4.1) (Bronneberg *et al.*, 2006). Unloaded

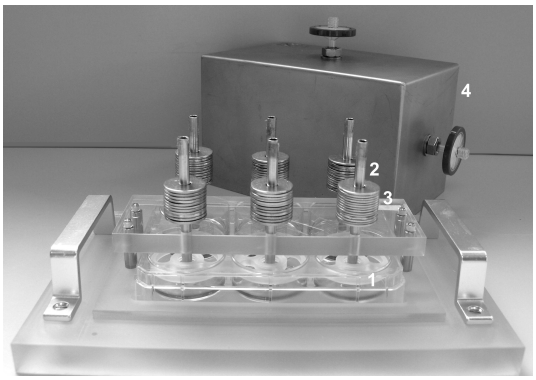


Figure 4.1: Custom-built loading device used to apply sustained pressures on six EpiDerm cultures simultaneously. Pressures were applied to EpiDerm cultures (1) using indenters (2) and additional weights (3). The grey box (4) is the lid of the loading device.

cultures were used as control. After each loading period, the culture supernatants were collected and stored at -80°C for later analysis of cytokines and chemokines, further referred to as 'medium cytokines and chemokines', as well as analysis of the cell membrane integrity. The EpiDerm cultures were gently washed with the provided phosphate buffered saline (PBS, MatTek Corporation, Ashland, MA, USA) and cut in two equal parts. One part was fixated for histological examination and the other part was used to determine the amount of cytokines and chemokines that remain inside the epidermal tissue, further referred to as 'culture cytokines and chemokines'. This part was placed in 450 μl of 1% (v/v) Triton X-100 (Merck Chemicals Ltd., Nottingham, UK) in hydrocortisone free maintenance medium at 4°C, according to recommendations from the supplier (MatTek Corporation, Ashland, MA, USA). After incubation for at least 8 h, the culture lysates were stored at -80°C for later analysis of the culture cytokines and chemokines. After multiplication by a factor two, the measured amount of culture cytokines and chemokines (pg) can be directly compared to the amount of medium cytokines and chemokines (pg).

4.2.3 Histological examination

The EpiDerm cultures were fixated in 10% phosphate-buffered formalin and processed for conventional paraffin embedding. The sections were cut at 5 μm and stained with haematoxylin and eosin (H&E). The tissue morphology was studied by light microscopy.

4.2.4 Membrane integrity

The cell membrane integrity (cell damage) of the EpiDerm cultures was measured by a colorimetric lactate dehydrogenase (LDH) assay (TOX-7, Sigma-Aldrich Co, St. Louis, Missouri, USA), according to recommendations from the supplier. This assay measures the membrane integrity as a function of the amount of cytoplasmic LDH released into the culture supernatants (Legrand *et al.*, 1992). Briefly, assay mixture was prepared by mixing equal amounts of LDH assay substrate, cofactor, and dye solutions. For all cultures, assay mixture was added to the culture supernatant in a proportion of two to one. After incubation for 30 min at room temperature in the dark, the color reaction was stopped by 1 M HCL. Plain medium was used as blank in this assay. Absorbance was determined at 490 nm using a plate reader. Background correction was performed at 650 nm.

4.2.5 Cytokines and chemokines

The levels of IL-1α, IL-1RA, IL-8, and TNF-α in the culture supernatants and culture lysates were determined by ELISA. TNF-α was quantified by a Quantikine high sensitive kit (HSTA00C, R&D systems, Abingdon, United Kingdom) according to the recommendations from the supplier. For IL-1α, IL-1RA, and IL8 (M9318, CLB, Amsterdam, the Netherlands) quantification, Maxisorp immunoplates (Nalge Nunc international, Roskilde, Denmark) were coated overnight at room temperature with capture antibody. Subsequently, the plates were blocked with PBS/0.5% BSA for 1 h at room temperature, after which the culture supernatants and standards were added to the plate. After incubation for 1 h at room temperature and washing (PBS/ 0.005% Tween-20), the detection antibody was added. The plates were, subsequently, incubated with 1:10000 diluted streptavidin-HRP (M2032, Sanquin Reagents, Amsterdam, The Netherlands) for a period of 30 min. The enzyme reaction was initiated by adding 0.2 mg/ml of o-phenylenediamine dihydrochloride in 0.11 M acetate pH 5.5 and 0.03% hydrogen peroxide and stopped with 2M H_2SO_4. Dilution buffer (PBS/0.5% BSA/0.005% Tween-20) was used as blank in this assay. Absorbance was determined at 490 nm using a plate reader. All antibodies and standards were purchased from R&D systems (Abingdon, United Kingdom), unless otherwise indicated.

4.2.6 Statistics

The results are expressed as mean and standard error of the mean (SEM). Weighted analyses of variance (ANOVA; SPSS 12.0.1, USA) were used to determine the effect of pressure and loading time on the amount of medium, culture, and total (i.e. medium+culture) cytokines and chemokines, as well as LDH. Dunnett's multiple comparisons test was used to compare the different loading times (2, 4, 6, 8, 16, and 24 h) to the 1 h loading time for both the loading (150 mmHg) and unloaded control group (0 mmHg). In addition, contrast was employed to compare the loaded and the unloaded control group to one another at each time point (1, 2, 4, 6, 8, 16, and 24 h). Differences were considered to be significant at a P-value$<$0.05.

4.3 Results

4.3.1 Damage assessment

Structural tissue damage was observed as a result of epidermal loading, unloaded cultures showed no damage (figure 4.2a). After 4 h of loading, the first signs of

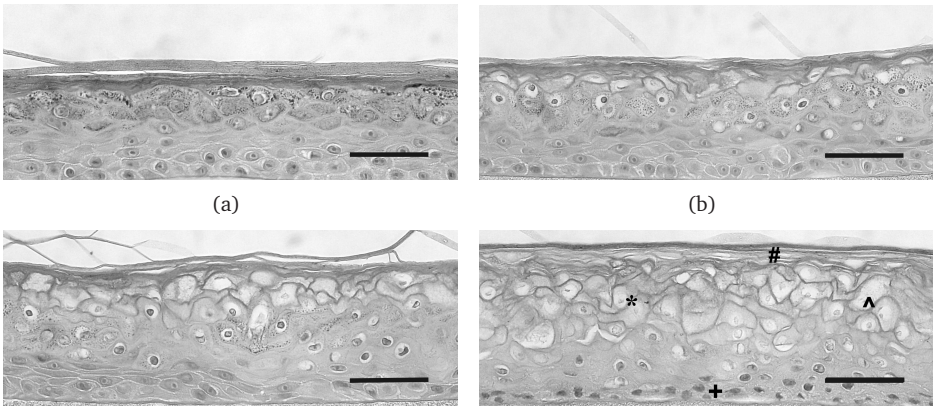
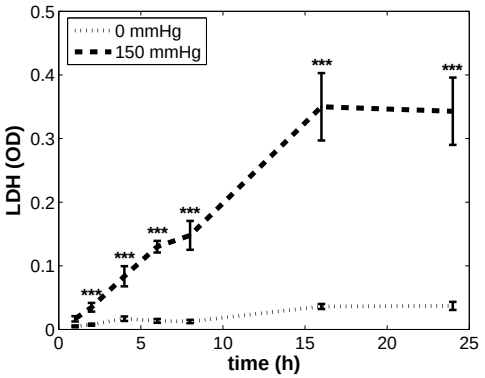


Figure 4.2: Histology of unloaded (0 mmHg) EpiDerm cultures (a) and of cultures loaded with 150 mmHg for 4 h (b), 8 h (c), and 24 h (d). The tissue structure of EpiDerm cultures that were loaded for 1 and 2 h are comparable to the unloaded control group (a). Furthermore, the tissue structure after 6 and 16 h of loading are comparable to images b and d, respectively. $*$ indicates cellular necrosis, $\wedge$ indicates cell swelling and vacuoles, # indicates disruption of stratum corneum, and + indicates compressed cells. The images were obtained in the middle of the loading region, and the bars indicate a distance of 50 μm.

structural tissue damage appeared in the upper layer of the epidermis (figure 4.2b). Some cell swelling, vacuoles, and necrosis could be detected just below the stratum corneum. Epidermal damage aggravated with increasing loading times. After 8 h of epidermal loading, more cell swelling and vacuoles could be detected (figure 4.2c). Furthermore, the stratum corneum appeared less compact and the stratum granulosum could no longer be distinguished. The most severe structural tissue damage was, however, found after 16 and 24 h of loading (figure 4.2d). In addition to the earlier observed structural tissue damage, cell swelling and vacuoles could also be detected in the middle layer of the epidermis. Furthermore, the keratinocytes in the lower layer of the epidermis appeared to be flattened in morphology.

Epidermal loading also affected the cell membrane integrity. Thus, an increase in the release of LDH was found in the culture supernatant when compared with the unloaded control group (0 mmHg) (figure 4.3). It is first worthy of note that the



(a)

Figure 4.3: LDH release (mean±SEM), expressed as optical density (OD) of the loaded (150 mmHg) and the unloaded control group (0 mmHg) at various time points (1, 2, 4, 6, 8, 16, and 24 h). *** indicates P<0.001 compared to the unloaded control group (0 mmHg).

release pattern of LDH in the unloaded control group varied minimally over the 24 h period. By contrast, a significant increase was detected after 2 h of loading (4.6-fold, P<0.001) and subsequent loading periods yielded increased differences compared to the unloaded control group. Following 16 h of loading the LDH release attained a maximum value (9.7-fold, P<0.001).

4.3.2 Medium cytokines and chemokines

The release of IL-1α, IL-1RA, IL-8, and TNF-α into the culture supernatant is almost comparable to the previously described release of LDH (figure 4.3 and 4.4). After only 1 h of loading, a significant increase in the release of IL-1α (4.7-fold vs 0 mmHg, P<0.01), IL-1RA (4.8-fold vs 0 mmHg, P<0.001), and IL-8 (3.6-fold vs 0 mmHg, P<0.01) was evident (figure 4.4a-c). The release of TNF-α was somewhat slower, with a significant increase only observed after 4 h of epidermal loading (5.1-fold vs 0 mmHg, P<0.05) (figure 4.4d). Furthermore, the levels of TNF-α in the culture supernatant were very small and could hardly be detected using a high sensitive immunoassay technique. For all cytokines and chemokines, the difference between the loading and unloading control group further increased with time. Following 16 h of loading, the release of IL-1α (5.1-fold, P<0.001), IL-1RA (11.7-fold, P<0.001), IL-8 (4.8-fold,(P<0.001) attained a maximum value. The release pattern of the unloaded control group only slightly increased in time, which was most evident for IL-1α and IL-8 (figure 4.4a and c).

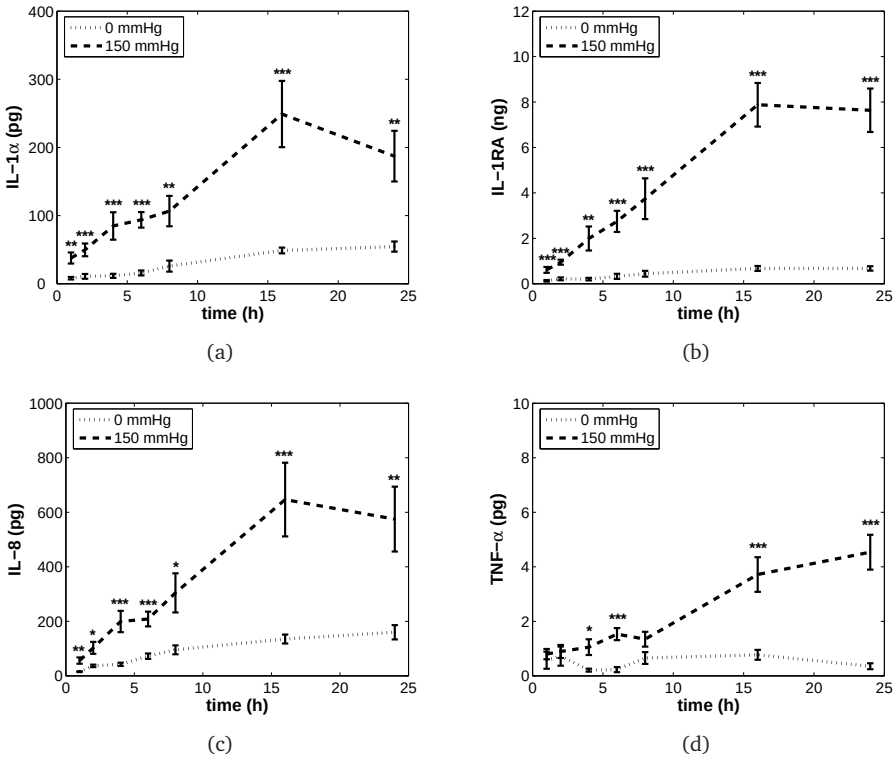


Figure 4.4: The amount (mean±SEM) of IL-1α (a), IL-8 (b), IL-1RA (c), and TNF-α (d) in the culture supernatant (medium cytokines and chemokines) after applying a pressure of 0 and 150 mmHg for various loading times (1, 2, 4, 6, 8, 16, and 24 h). * indicates P<0.05, ** indicates P<0.01, and *** indicates P<0.001 compared to the unloaded control group (0 mmHg).

4.3.3 Culture cytokines and chemokines

Compared to the unloaded control group, epidermal loading did not change the amount of IL-1α, IL-1RA, IL-8, and TNF-α inside the EpiDerm cultures (i.e. culture cytokines and chemokines) (data not shown). To determine whether cytokines and chemokines were stored intracellularly prior to loading or were produced on demand, the total amount of cytokines and chemokines was calculated from the sum of the amounts in the medium and the cultures. Figure 4.5 shows the amounts of medium, culture and total cytokines and chemokines in time during loading with 150 mmHg. The total amount of IL-1α remained nearly constant with time (figure 4.5a). The total amount of IL-1RA increased after 6 h of loading (P<0.05) and again decreased after 16 h (P<0.01) and 24 h (P<0.01) as compared to the 1 h loading group (figure 4.5b).

Furthermore, these cytokines seemed to be stored intracellularly, since the amount of IL-1α and IL-1RA inside the epidermal tissue is considerably higher than that in the culture supernatants for all time periods (figure 4.5a-b). On the other hand, IL-8 and TNF-α seemed to be produced as a result of mechanical loading, since the medium amount exceeded the culture amount with increasing loading times (figure 4.5c-d). This trend was most evident for TNF-α. Furthermore, an increasing trend in the total amount of IL-8 and TNF-α was observed with loading periods.

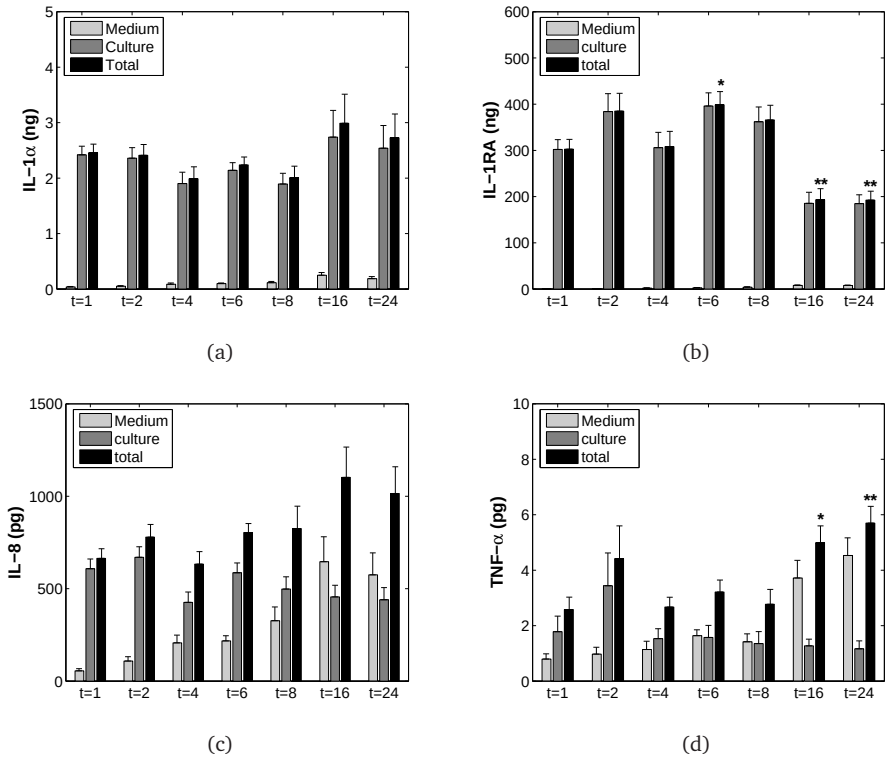


Figure 4.5: The medium, culture, and total amount (mean+SEM) of IL-1α (a), IL-8 (b), IL-1RA (c), and TNF-α (d) after applying a pressure of 150 mmHg for various loading times (1, 2, 4, 6, 8, 16, and 24 h). The total amount of cytokines and chemokines after 2, 4, 6, 8, 16, and 24 h are compared to the total amount after 1 h of loading. * indicates $P<0.05$ ** indicates $P<0.01$ compared to the unloaded control group (0 mmHg).

4.4 Discussion

Cytokines and chemokines that can be measured *in vitro* when tissue damage is barely visible, are of interest in this study, since they might be suitable for early detection of pressure ulcer *in vivo*. The aim of the present study is to evaluate the pressure-time regulation of cytokines and chemokines (IL-1α, IL-1RA, IL-8, and TNF-α). Epidermal equivalents were, therefore, subjected to either 0 or 150 mmHg in a custom-built loading device for various loading periods (1, 2, 4, 6, 8, 16 and 24 h).

In the present study, commercially available EpiDerm cultures were used. These epidermal equivalents provide an ideal model to evaluate biochemical markers upon mechanical loading. Since, the general morphology of the EpiDerm cultures is comparable to that of normal human epidermis; all epidermal strata (i.e. stratum basale, stratum spinosum, stratum granulosum, and stratum corneum) are present in these cultures (Netzlaff *et al.*, 2005; Ponec *et al.*, 2002). Besides, epidermal equivalents, such as EpiDerm, are currently employed as diagnostic models for *in vitro* toxicology testing (Bernhofer *et al.*, 1999; Coquette *et al.*, 2003; Perkins *et al.*, 1999; Faller and Bracher, 2002; Faller *et al.*, 2002). Cytokines and chemokines were used as screening markers in these studies. Furthermore, the observed tissue damage and the release of cytokines and chemokine into the culture supernatants after 24 h of loading is comparable with an earlier study in chapter 3. This study indicated that reproducible results can be obtained using the current model system.

The first changes in tissue architecture appeared after 4 h of epidermal loading with 150 mmHg. Some cell swelling, vacuoles, and necrosis could be detected just below the stratum corneum. Cell swelling or cytoplasmic swelling is known to be one of the first signs of cell damage (Cobb *et al.*, 1996). This structural tissue damage increased with loading times. After 8 h, the stratum corneum seemed to be affected as well, and the stratum granulosum could no longer be distinguished. The stratum corneum represents the epidermal permeability barrier, that protects the skin against excessive water loss as well as mechanical and chemical insults (Elias and Friend, 1975). A less compact stratum corneum might, therefore, indicate loss of structure and function of the epidermal barrier. Severe tissue damage was observed after loading the EpiDerm cultures for 16 and 24 h. In addition to the earlier observed structural tissue damage, cell swelling and vacuoles could also be detected in the middle layer of the epidermis and the lower layer seemed to be compressed.

In a normal situation, IL-1α is only lost from the epidermis by desquamation of the stratum corneum, due to the lack of a hydrophobic leader sequence for transmembrane secretion (Uchi *et al.*, 2000). However, chemical exposure, UV irradiation, tape stripping, as well as sustained mechanical loading are known to disrupt the cell membrane and release a large amount of IL-1α from the cells (Bernhofer *et al.*, 1999; Bronneberg *et al.*, 2006, 2007; Faller and Bracher, 2002; Flint *et al.*, 1998; Nickoloff and Naidu, 1994; Oxholm *et al.*, 1988; Wood *et al.*, 1992, 1997). IL-1α is a pluripotent, multifunctional cytokine that plays a key role in the initiation and development of inflammatory and immune responses (Uchi *et al.*, 2000). Keratinocytes maintain prodigious intracellular stores of IL-1α in the epidermis. Furthermore, this cytokine is known to induce the production of other cytokines and chemokines, such as IL-8

and TNF-α (Enk and Katz, 1992; Uchi *et al.*, 2000). IL-1RA, which is also constitutively produced by keratinocytes in the epidermis, is known to inhibit the activity of IL-1α. In the present study, an increase in IL-1α, IL-1RA, IL-8 could already be found in the culture supernatant, before the onset of visible tissue damage (i.e. after 4 h of epidermal loading). This increase was first apparent as quickly as 1 h after epidermal loading. A rapid increase in the release of LDH could also be observed, which confirms that the cell membrane integrity was indeed affected. The release of TNF-α was somewhat slower, since an increase was only found when the first signs of tissue damage were already apparent. Besides, only very small levels of TNF-α were present in the culture supernatant, which could hardly be detected using a 'high sensitive' immunoassay technique.

Upon epidermal loading with 150 mmHg, the release of IL-1α, IL-1RA, and IL-8 into the culture supernatant showed a monotonic increase until approximately 16 h of loading. The release of TNF-α seemed to increase until 24 h of loading. During loading, the amount of culture cytokines and chemokines remained nearly constant in time. These results are in agreement with the observed structural tissue damage, as well as with the extent of cell membrane disruption or LDH leakage. Both damage assessment methods showed an increase in tissue damage until a maximum was approximately reached after 16 h of loading. An increase in tissue damage with loading periods was already found in earlier studies on pressure ulcer development (Breuls *et al.*, 2003).

The total amount (i.e. sum of the culture and medium amounts) of IL-1α remained nearly constant in time during loading. This finding might indicate that no additional production of IL-1α took place. At the same time, a decrease was, however, observed for the total amount of IL-1RA after 16 and 24 h of loading. Since, no difference was found between the loading (150 mmHg) and unloading (0 mmHg) groups, this decrease might be caused by degradation of this protein in time. The current study, furthermore, confirms that both IL-1α and IL-1RA are stored in the epidermis (Uchi *et al.*, 2000), since a large difference was found between the culture and medium amounts. On the other hand, the medium amount of IL-8 and TNF-α even exceeded the culture amount with increasing loading times. An increasing trend in the total amount of IL-8 and TNF-α was subsequently observed as well. These results indicate that IL-8 and TNF-α were produced on demand during epidermal loading.

In conclusion, cytokines and chemokines, such as IL-1α, IL-1RA and IL-8, seem promising biochemical markers for early detection of superficial pressure ulcers. After 1 h of epidermal loading and before the onset of structural tissue damage, a first increase in the release of IL-1α, IL-1RA and IL-8 could be observed in the culture supernatant as compared with the unloaded control group. This fast release did not seem to be solely dependent on the presence of intracellular stores in the epidermis (IL-1α and IL-1RA). Since, an increase in IL-8, which is produced on demand upon skin irritation or injury, could also be detected after this 1 h loading period. On the other hand, TNF-α does not seem to be a suitable biochemical marker for early detection of mechanically-induced epidermal damage, since this cytokine was only raised when the first signs of tissue damage were already apparent. Furthermore, the amount of TNF-α was very small and could hardly be detected using a 'high sensitive'

immunoassay technique. Currently, a clinical study is being performed to determine whether the level of IL-1α, IL-1RA, and IL-8 is actually raised in patients with grade I ulcers. For this purpose, a simple detection technique, based on Perkins *et al.* (2001), is used for measuring cytokines and chemokines at the skin surface. A non-invasive method for monitoring the tissue status and determining the risk for pressure ulcer development is of utmost importance, particularly in clinical situations.

4.5 Acknowledgements

The authors would like to thank Kang-Yuen Rosaria-Chak, for performing the TNF-α analyses. This work was financially supported by SenterNovem, an agency from the Ministry of Economic Affairs in the Netherlands.

Chapter 5

The effect of synthetic urine on mechanically-induced epidermal damage

The contents of this chapter are based on D. Bronneberg, L. H. Cornelissen, C. W. J. Oomens, F. P. T. Baaijens and C. V. C. Bouten (2007) *The effect of synthetic urine on mechanically-induced epidermal damage*, Skin Research and Technology, submitted.

5.1 Introduction

Pressure ulcers are areas of soft tissue breakdown that result from sustained mechanical loading of the skin and underlying tissues (APUAP, 1998). Urinary incontinence is widely recognized as an important risk factor for the formation of pressure ulcers. Worldwide, there are over 200 million people with significant urinary incontinence and many more with mild bladder problems (Ersser *et al.*, 2005). The precise relation between pressure ulcers and urinary incontinence is, however, still unknown. There are almost no studies to quantitatively determine the individual and combined effects of mechanical loading and incontinence on pressure ulcer development. This lack of scientific evidence is problematic, considering the high prevalence of pressure ulcers in the Netherlands (Bours *et al.*, 2002). At this moment, there is an ongoing debate in the literature on the usefulness of incontinence as risk factor, and on the difference between incontinence lesions and pressure ulcers (Krause *et al.*, 2005; Sharp and McLaws, 2006; Houwing *et al.*, 2007). It is therefore of topical importance to examine the relation between pressure ulcers and urinary incontinence in more detail.

Tsai and Maibach (1999) demonstrated that ongoing exposure to water can already cause skin damage and impairment of its barrier function. However, urine contains many more potentially irritating substances than water alone. It contains ammonia that raises the pH, and urea that is converted to ammonia in the presence of skin bacteria (Gray, 2004; Berg *et al.*, 1994). Raising the pH of the skin might further affect the barrier function by interfering with the production of mildly acidic lipids, the production of necessary enzymes and the process of keratinization critical to damage repair. The normal pH of the skin ranges from pH 5.4 to pH 5.9 (Ersser *et al.*, 2005) but, in the presence of urine, values in excess of pH 8 are evident (Gray, 2004), which might have implications for cell viability (Taylor, 1962).

Previous studies in chapter 3 demonstrated that sustained mechanical loading of the epidermis (i.e. the superficial layer of the skin) with 0-200 mmHg for 24 h increased the extracellular release of cytokines and chemokines (IL-1α, IL-1RA, IL-8, and TNF-α) (Bronneberg *et al.*, 2007). Commercially available epidermal equivalents (EpiDerm), of which the general morphology is comparable to human epidermis, were used in these studies (Cannon *et al.*, 1994; Netzlaff *et al.*, 2005). After 1 h of loading with 150 mmHg and before the onset of structural tissue damage, a first increase in the release of IL-1α, IL-1RA, and IL-8 was observed (chapter 4). Lee *et al.* (1997) and Takei *et al.* (1998) also showed that mechanical straining of keratinocyte monolayers promotes the expression and release of IL-1α and IL-1RA. Therefore, cytokines and chemokines seem promising markers for early detection of superficial pressure ulcers.

It is hypothesized that urine exposure makes the skin, in particular the epidermis, more susceptible to sustained mechanical loading. In the present study, it is determined whether the amount of mechanically-induced epidermal damage increases when pretreated with urine. Therefore, EpiDerm cultures were either statically cultured or exposed to synthetic urine (s-urine) for 4 h, prior to applying various pressures (0, 50, 75, 100 mmHg) for 24 h. The formulation of s-urine that is used in

the current study contains the main constituents of human urine (i.e water, urea, inorganic salts) (Ersser *et al.*, 2005). The pH of the s-urine was set to pH 9 to simulate the conversion of urea into ammonia, which acts as a base in aqueous solutions. Early tissue damage was assessed by the release of objective markers (i.e. IL-1α and IL-1RA) in the culture supernatant. In addition, the cell membrane integrity and the tissue structure of the EpiDerm cultures were studied after loading, to further increase insight into the damage process.

5.2 Materials and methods

5.2.1 Engineered epidermal equivalent

A commercially available human epidermal equivalent, EpiDerm (EPI-200, MatTek Corporation, Ashland, MA, USA), was used as an *in vitro* model of the epidermis in this study (Cannon *et al.*, 1994). These circular EpiDerm cultures (diameter=8 mm) are comprised of human-derived epidermal keratinocytes, which have been cultured on standing cell culture inserts (Millipore, Billerica, MA, USA) at the air-liquid interface to form a multilayered, differentiated model of the human epidermis (Cannon *et al.*, 1994).

Upon receipt, the EpiDerm cultures were placed in 6-well plates and cultured overnight at 37°C and 5% CO_2 in 900 μl/well of hydrocortisone free maintenance medium (EPI-100-MM-HCF, MatTek Corporation, Ashland, MA, USA). The next morning, the cultures were transferred to fresh medium and directly used in a loading experiment. Two separate batches of EpiDerm cultures (n=24) were used.

5.2.2 Exposure to s-urine

The composition of synthetic urine (s-urine), which is used in the current study, was based on Mayrovitz and Sims (2001). S-urine was prepared by dissolving urea (25 g/l), disodium hydrogen orthophosphate (anhydrous) (2.5 g/l), sodium chloride (NaCL, 8.4 g/l), ammonium chloride (3 g/l), creatinine (2 g/l), and sodium sulfite (anhydrous) (1.5 g/l) in ultrapure water. The pH level of s-urine was adjusted to pH 9 using 8 M NaOH. Addition of NaOH increases the osmolarity of a solution. To generate s-urine pH 9 with a similar osmolarity as that of Mayrovitz and Sims (2001), less sodium chloride (NaCL) was added (8.4 g/l versus 9 g/l). All constituents of s-urine were supplied by Sigma-Aldrich Chemie B.V. (Zwijndrecht, NL).

EpiDerm cultures (+U) were loaded with 100 μl of s-urine pH 9 for a period of 4 h. Unexposed cultures (-U) were used as control. Incubation of these cultures at 37°C and 5% CO_2 slightly decreased the pH of s-urine; after 4 h the pH dropped to 8.5. At the end of the incubation period, the EpiDerm cultures were washed three times with phosphate buffered saline (PBS, MatTek Corporation, Ashland, MA, USA) to remove any residual s-urine. Subsequently, the EpiDerm cultures were mechanically loaded for an additional 24 h.

5.2.3 Exposure to pressure

After static culture (-U) or exposure to s-urine (+U) for 4 h, pressures of 0, 50, 75, and 100 mmHg were applied to the top surface of the EpiDerm cultures for an additional period of 24 h using a custom-built loading device (chapter 2-4). In short, indenters (diameter=5 mm) and additional weights were used for applying the specified pressures on top of the cultures.

After loading, the culture supernatants were collected and stored at -80°C for subsequent analysis of extracellular IL-1α and IL-1RA, as well as analysis of cell membrane integrity. The EpiDerm cultures were gently washed with the provided PBS and fixated for histological examination.

5.2.4 IL-1α and IL-1RA

The levels of IL-1α and IL-1RA, were determined in the culture supernatants by ELISA. Maxisorp immunoplates (Nalge Nunc international, Roskilde, Denmark) were coated overnight at room temperature with capture antibody (R&D systems, Abingdon, United Kingdom): 2 μg/ml of mouse antihuman IL-1α (MAB200) and 2.5 μg/ml of mouse antihuman IL-1RA (MAB280). Subsequently, the plates were blocked with PBS/0.5% BSA for 1 h at room temperature, after which the culture supernatants and standards were added to the plate: 7.8-1000 pg/ml recombinant human IL-1α (200-LA-002) and 31.3-4000 pg/ml recombinant human IL-1RA (280-RA-010). After incubation for 1 h at room temperature and washing (PBS/ 0.005% Tween-20), the detection antibody was added: 12.5 ng/ml biotinylated goat antihuman IL-1α (BAF-200) and 200 ng/ml biotinylated goat antihuman IL-1RA (BAF280). The plates were subsequently incubated with 1:10000 diluted streptavidin-HRP (M2032, Sanquin Reagents, Amsterdam, The Netherlands) for a period of 30 min. The enzyme reaction was initiated by adding 0.2 mg/ml of o-phenylenediamine dihydrochloride in 0.11 M acetate pH 5.5 and 0.03% hydrogen peroxide and stopped with 2M H_2SO_4. Absorbance was determined at 490 nm using a plate reader. All antibodies and standards were purchased from R&D systems (Abingdon, United Kingdom).

5.2.5 Membrane integrity

The cell membrane integrity (cell damage) of the EpiDerm cultures was measured by a colorimetric lactate dehydrogenase (LDH) assay (TOX-7, Sigma-Aldrich Co, St. Louis, Missouri, USA), according to recommendations from the supplier. This assay measures the membrane integrity as function of the amount of cytoplasmic LDH released into the culture supernatants (Legrand *et al.*, 1992). Briefly, assay mixture was prepared by mixing equal amounts of LDH assay substrate, cofactor and dye solutions. For all cultures, assay mixture was added to the culture supernatant in a proportion of two to one. After incubation for 30 min at room temperature in the dark, the color reaction was stopped by 1 M HCL. Absorbance was determined at 490 nm using a plate reader. Background correction was performed at 650 nm.

5.2.6 Histological examination

EpiDerm samples were fixed in 10% phosphate-buffered formalin and processed for conventional paraffin embedding. The sections were cut at 5 μm and stained with haematoxylin and eosin (H&E). The tissue morphology was studied by light microscopy.

5.2.7 Statistics

In the current study, EpiDerm cultures were either statically cultured (-U) or exposed to s-urine (+U) for 4 h prior to applying various pressures (0, 50, 75, and 100 mmHg) for 24 h. Thus, 8 different experimental groups can be distinguished in this study: -U0, +U0, -U50, +U50, -U75, +U75, -U100, and +U100. The results of IL-1α, IL-1RA, and LDH of the different experimental groups (n=6) are expressed as mean + standard error of the mean (SEM). Analyses of Variance (ANOVA; SPSS 12.0.1,USA) were used to determine whether the factors pressure and exposure to s-urine interact in a synergistic way. Student's T-tests were performed to determine the effect of s-urine on the release of IL-1α, IL-1RA, and LDH during loading (0, 50, 75, and 100 mmHg) (figure 5.1 and 5.2). Therefore, the experimental groups -U0 and +U0 were compared to one another, as well as the groups -U50 and +U50, -U75 and +U75, and -U100 and +U100. Furthermore, ANOVA analyses followed by a Dunnet's multiple comparison test were performed on the groups -U0, -U50, -U75, and -U100 to determine the effect of pressure on the release of IL-1α, IL-1RA, and LDH.

5.3 Results

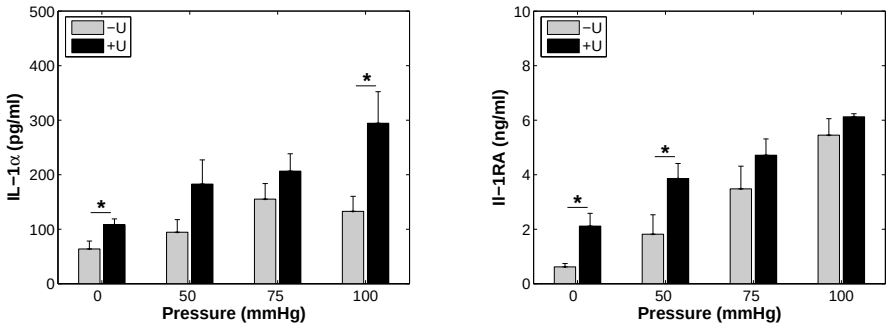
5.3.1 Effect of s-urine

Exposure to s-urine caused epidermal damage. When no pressure was applied (+U0) to the top surface of the EpiDerm cultures, s-urine increased the extracellular release of IL-1α (1.7-fold, $P<0.05$), IL-1RA (3.4-fold, $P<0.05$), and LDH (1.9-fold, $P<0.05$) (+U0 versus -U0) (figure 5.1 and 5.2).

Furthermore, histological analysis revealed that s-urine induced wrinkles in the stratum corneum of the EpiDerm cultures (figure 5.3b). These wrinkles might indicate that the barrier function of the epidermis, which resides in the stratum corneum, is affected.

5.3.2 Effect of pressure

Epidermal damage was also observed when sustained pressures were applied on the top surface of the EpiDerm cultures. The degree of epidermal damage increased with rising pressures. A first significant increase in the extracellular release of damage markers, such as IL-1α (2.4-fold, $P<0.05$) and IL-1RA (5.6 fold, $P<0.01$), was detected at the relatively low pressure of 75 mmHg (-U75 versus -U0) (figure 5.1) when tissue damage was barely visible. Compared with the unloaded control group



(a) (b)

Figure 5.1: IL-1α (a) and IL-1RA (b) release (mean+SEM) of EpiDerm cultures that were either statically cultured (-U) or exposed to s-urine (+U) for 4 h, prior to applying various pressures for 24 h. * indicates $p<0.05$.

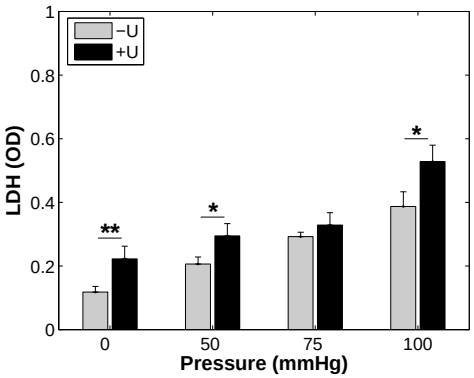


Figure 5.2: LDH release (mean+SEM), expressed as optical density (OD), of EpiDerm cultures that were either statically cultured (-U) or exposed to s-urine (+U) for 4 h, prior to applying various pressures for 24 h. * indicates $p<0.1$ and ** indicates $p<0.05$.

(-U0), the stratum granulosum could no longer be distinguished at a pressure of 75 mmHg (-U75) (figure 5.3c). At a pressure of 100 mmHg (-U100) deleterious changes were found in the tissue architecture, such as loosening of the stratum corneum, cell swelling, increased number of vacuoles, and some evidence of necrosis (figure 5.3e). Furthermore, the cell membrane integrity appeared to be affected as a result of sustained mechanical loading, since an increase in the release of LDH (-U75 versus -U0, 2.5-fold, $P<0.01$) was found with rising pressures (figure 5.2).

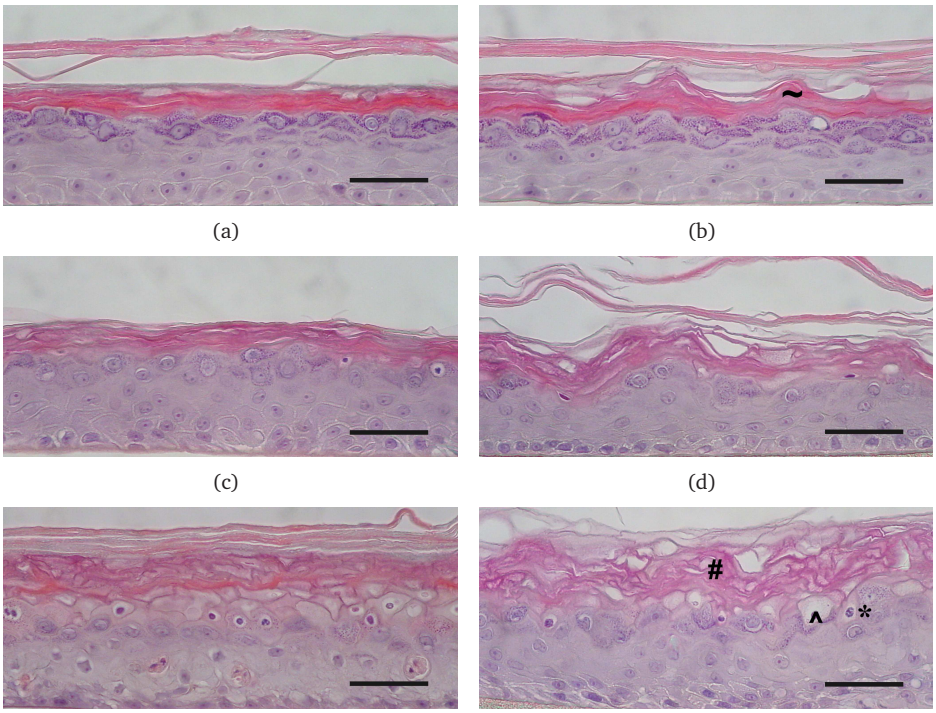


Figure 5.3: Epidermal histology of EpiDerm cultures that were either statically cultured (-U) or exposed to s-urine (+U) for 4 h, prior to applying various pressures (0, 50, 75, 100 mmHg) for 24 h. Images are shown of the experimental groups -U0 (a), +U0 (b), -U75 (c), +U75 (d), -U100 (e), and +U100 (f). The experimental groups -U50 and +U50 are comparable to -U75 (c) and +U75 (d), respectively. $\sim$ indicates wrinkles, $*$ indicates cellular necrosis, $\wedge$ indicates cell swelling and vacuoles, and # indicates disruption of stratum corneum. The images were obtained in the middle of the loading region, and the bars indicate a distance of 50 μm.

5.3.3 Combined effect of s-urine and pressure

Exposure to both s-urine and pressure appeared to further increase epidermal damage, although no synergistic interaction was found between both factors. Nevertheless, a cumulative increase in the release of IL-1α, IL-1RA, and LDH could, roughly, be observed when the EpiDerm cultures were exposed to s-urine prior to applying various pressures (-U50 versus +U50, -U75 versus +U75, and -U100 versus +U100) (figure 5.1 and 5.2). This increase was, however, not statistically significant for each pressure group, although a clear trend was visible. The highest release in IL-1α, IL-

1RA, and LDH was observed after loading the EpiDerm cultures with both s-urine and 100 mmHg (+U100). This experimental group also showed the most severe structural tissue damage; wrinkles could be detected and the different layers of the epidermis could no longer be distinguished from one another. Furthermore, loosening of the stratum corneum, cell swelling, vacuoles, and necrosis could be observed (figure 5.3f). In general, the structural tissue damage that appeared after applying both s-urine and pressure (figure 5.3d and f) corresponded to the sum of the damage induced by either factors alone.

5.4 Discussion

Urinary incontinence is widely recognized as an important risk factor for pressure ulcer formation, despite the lack of scientific proof for a causal relation (Krause *et al.*, 2005). We hypothesize that exposure to urine makes the skin, in particular the epidermis, more susceptible to sustained mechanical loading. In the present study, we want to determine whether the amount of mechanically-induced epidermal damage increases upon pretreatment with urine. Therefore, epidermal equivalents (EpiDerm) were either statically cultured (-U) or exposed to s-urine (+U) for 4 h prior to applying various pressures (0, 50, 75, and 100 mmHg) for 24 h. IL-1α and IL-1RA were used as objective markers for early epidermal damage (chapter 3 and 4). The cell membrane integrity (LDH release) and tissue structure (histology) were studied as well, to further increase insight in the epidermal damage process.

The commercially available EpiDerm cultures, that are used in the current study, provide a good model system to investigate the effects of s-urine and pressure *in vitro*. Since, the general morphology of the EpiDerm cultures is comparable to that of normal human epidermis. All epidermal strata (i.e. stratum basale, stratum spinosum, stratum granulosum, and stratum corneum) are present in these cultures (Netzlaff *et al.*, 2005; Ponec *et al.*, 2002). As in native epidermis, the cells in the stratum basale appear columnar to round, whereas in the stratum spinosum the cells are flattened (Netzlaff *et al.*, 2005). The lamellar bodies in the stratum granulosum appear normal, and the keratohyalin granules appear rounded to satellite in shape. Furthermore, it was shown that the stratum corneum of the EpiDerm cultures exhibit a normal basket-weave pattern and that the extrusion of the lamellar body contents at the interface between the stratum granulosum and stratum corneum is complete (Ponec *et al.*, 2002). Furthermore, the epidermal damage that was observed in the current study, as a result of sustained mechanical loading, is comparable to earlier studies (chapter 2-4). This indicates that reproducible results can be obtained with the current model system.

Exposure to s-urine (+U0) affected the stratum corneum, since wrinkles were apparent in the uppermost layer of the epidermis. Furthermore, an increase in the release of damage markers (i.e. IL-1α and IL-1RA) and LDH was observed. The stratum corneum normally provides a barrier to water loss and avoids the penetration of harmful substances into the skin (Williams, 2001). This protective layer consists of flattened dead cells which are called corneocytes and are filled with the protein

keratin (Ersser *et al.*, 2005). These corneocytes are packed tightly together in layers between which lipid bilayers are secreted. Further stability to the barrier structure is provided by the ability of the corneocytes to retain water, causing them to swell and close together tightly. The normal pH of the epidermis ranges between pH 5.4 and pH 5.9. This 'acid mantle' provides resistance to microbial invasion. Warner *et al.* (2003) already demonstrated that sustained exposure to urine can induce extensive swelling of the stratum corneum and lead to disruption of the barrier structure.

Water accounts for about 95% of the total volume of urine. The remaining 5% consists of organic salts derived from cellular metabolism, with urea being the major component and inorganic salts principally comprising of sodium, chloride and potassium ions, sulphate, phosphate and ammonium salts (Ersser *et al.*, 2005). S-urine, which is used in the present study, contains the main constituents of urine (Mayrovitz and Sims, 2001). Mayrovitz and Sims (2001) demonstrated *in vivo* that skin wetted with s-urine exhibited a significant decrease in skin hardness, temperature, and blood flow during pressure loading when compared to dry sites. The pH of normal urine ranges between pH 5.5 and pH 6.5, but in the presence of bacteria at the skin surface, urea is converted to ammonia and the pH of urine can even raise above pH 8 (Gray, 2004; Berg *et al.*, 1994). To mimic urinary incontinence with high accuracy, the pH of s-urine was set to pH 9 in the current study, while the osmolarity of the solution was kept constant. Exposure to a high pH is already known to affect the barrier properties of the epidermis (Matoltsy *et al.*, 1968), as well as the cell viability (Taylor, 1962). A pilot study by our group demonstrated that the viability of a monolayer of keratinocytes drastically decreased when the pH of the culture medium was raised from pH 7.5 to pH 9 for a period of 24 h (data not shown). To maintain the pH of the culture medium buffered with bicarbonate at a value of 9, the keratinocytes were cultured without CO_2 in this pilot study. Since, CO_2 can alter the pH of culture medium according to the Henderson-Hasselbach equation (Taylor, 1962).

$$pH = pKa + log((HCO_3^-)/(CO_2)) \tag{5.1}$$

In this equation pKa is a constant, (CO_2) the molar concentration of CO_2, and ($HCO_3{}^-$) the molar concentration of bicarbonate ion in the culture medium. In contrast to this pilot study, the EpiDerm cultures of the current study were incubated at 37°C and 5% CO_2. The concentration of CO_2 was not set to 0%, since an absence of CO_2 might influence cellular processes. Furthermore, a 4 h exposure time was chosen, to keep the pH of s-urine nearly constant.

Exposure to both s-urine and pressure seems to further increase epidermal damage, although no synergistic interaction was found between both factors. A cumulative increase in the release of IL-1α, IL-1RA, and LDH could, roughly, be observed. Furthermore, it seems that the structural tissue damage that appeared after applying both s-urine and pressure also corresponds well to the sum of the damage induced by either factors alone. Last, the most severe epidermal damage was observed after applying both s-urine and the highest pressure of 100 mmHg. Future studies will focus on the effect of s-urine on epidermal tissue that has already been exposed to pressure, in addition to applying s-urine prior to mechanical loading.

In conclusion, pretreatment with s-urine makes the epidermis more susceptible to

sustained mechanical loading and increases the amount of mechanically-induced epidermal damage. A cumulative increase in epidermal damage was observed when both s-urine and pressure were applied. Although these objective findings are obtained *in vitro*, they imply that urinary incontinent patients have a higher risk of developing pressure ulcers. Therefore, this study justifies the use of urinary incontinence as a risk factor for pressure ulcers.

5.5 Acknowledgements

The authors would like to thank Jolet Sonsma for performing the *in vitro* experiments. This work was financially supported by SenterNovem, an agency from the Ministry of Economic Affairs in the Netherlands.

Chapter 6

In vivo detection of grade I pressure ulcers

6.1 Introduction

Pressure ulcers are areas of soft tissue breakdown resulting from sustained mechanical loading of the skin and underlying tissues (APUAP, 1998). They are painful, difficult to treat, and represent a burden to the community in terms of health care and finances (Bouten *et al.*, 2003b). The prevalence of pressure ulcers is unacceptably high and varies from 13% in hospitalized patients to 35% in institutions for the physically handicapped (Bours *et al.*, 2002). This high prevalence is partly due to limited risk assessment and detection techniques. Currently, risk assessment is mainly performed by questionnaires or scales like the Norton (Norton, 1989), Braden (Bergstrom *et al.*, 1987), Waterlow (Waterlow, 1985), and CBO (CBO, 2002). Most risk assessment scales are based on expert opinion or literature review and have a limited scientific background (Schoonhoven *et al.*, 2002). Furthermore, Schoonhoven *et al.* (2002) demonstrated that risk assessment scales do not predict the degree of susceptibility with the required accuracy and indeed, up to 30% of the hospitalized patients with pressure ulcers are still misclassified. Accordingly, patients with high risk might not receive adequate preventive measures. The Dutch National Prevalence Survey demonstrated that grade I ulcers, the first stage of superficial ulcers in skin, accounted for approximately 50% of the prevalence of pressure ulcers (Bours *et al.*, 2002). Grade I ulcers are classified as non-blanchable erythema (NBE) of intact skin (Defloor *et al.*, 2005). Currently, the transparant disk method is commonly used for visual observation of NBE, in which the disk is pressed into the erythematous tissue. If the skin under this disk does not blanch, it is regarded as a grade I ulcer (Halfens *et al.*, 2001; Vanderwee *et al.*, 2006). For individuals with darker skin types, it is more difficult to determine grade I ulcers. Alternative measures, such as skin temperature, edema, induration or skin hardness might be used in these individuals. Nonetheless, Rosen *et al.* (2006) demonstrated that patients with darker skin types are still less likely to have their grade I ulcers identified compared to patients with lighter skin types. More importantly, the mentioned technique aims for measuring the consequences of 'harm' already done to the skin (i.e. inflammation). Whereas pressure ulcer prevention should aim for earlier detection of developing ulcers. Improved pressure ulcer risk assessment is, therefore, necessary. A first step in developing a new risk assessment tool is directed towards the detection of skin reactions that precede grade I ulcers. Cytokines and chemokines are of interest for pressure ulcer detection, since they are known to mediate inflammatory responses.

Perkins *et al.* (2001) developed a non-invasive method to absorb cytokines and chemokines, such as interleukin 1 α (IL-1α), interleukin 1 receptor antagonist (IL-1RA), and interleukin 8 (IL-8), from the skin surface using Sebutape. It is a lipophilic polymeric silicone coated film, which has been used by many investigators in clinical studies for the extraction of sebum from the skin (Pagnoni *et al.*, 1994). These tapes cause minimal stripping of corneocytes or damage to the stratum corneum, and inflict minimal pain to the patient. The precise role of cytokines and chemokines in the development of pressure ulcers is still unknown. Previous studies, however, demonstrated that IL-1α is a pluripotent, multifunctional cytokine that plays a key role in the initiation and development of inflammatory and immune responses (Uchi

et al., 2000; Kupper, 1990; Luger, 1989). Keratinocytes constitutively produce IL-1α and their competitive antagonist, IL-1RA. Furthermore, IL-1α is known to induce the production of IL-8. In addition, cytokines and chemokines have widely been used as screening parameters for measuring skin reactivity to various chemicals as well as UV irradiation (Bernhofer *et al.*, 1999; Faller and Bracher, 2002; Flint *et al.*, 1998; Nickoloff and Naidu, 1994; Oxholm *et al.*, 1988). Furthermore, studies by our group demonstrated increased levels of IL-1α, IL-1RA, and IL-8 upon mechanical loading of an epidermal model system for pressure ulcer development (chapter 3 and 4). For example an increase could be observed after 1 h of loading at a pressure level of 150 mmHg. Therefore, IL-1α, IL-1RA, and IL-8 might represent suitable markers for the early and objective detection of mechanically-induced skin damage, such as pressure ulcers.

The aim of the present study is to examine cytokines and chemokines, such as IL-1α, IL-1RA, and IL-8, in grade I ulcers *in vivo*. This study was conducted at the Catharina Hospital in Eindhoven, the Netherlands. Sebutapes were used to collect cytokines and chemokines from the skin surface of patients. This study was designed to address the following questions:

- Is the level of cytokines and chemokines raised at a skin site (i.e. sacrum) that is mechanically loaded upon supine lying and sitting?
- Is there a difference in cytokines and chemokines at the site of a grade I ulcer compared to visually intact skin of patients?

6.2 Materials and methods

6.2.1 Study population

This study was conducted at the Catharina Hospital in Eindhoven from January until April 2007. Based on pressure ulcer prevalence figures, three different wards were involved in this study; the general surgical, the orthopaedic, and lung disease wards. Patients with grade I ulcers at the sacrum were included. In addition, patients without pressure ulcers served as control group. Grade I ulcers were assessed by the clinical nursing staff using the transparant disk method, to identify non-blanchable erythema (Halfens *et al.*, 2001; Vanderwee *et al.*, 2006). Patients with skin conditions, such as eczema and psoriasis, were excluded. A total of 36 patients participated in this study, which were evenly distributed over the three different wards. The composition of the study population is indicated in tabel 6.1. The research protocol was approved by the Medical Ethics Committee of the Catharina Hospital. Furthermore, all patients signed informed consent forms prior to participation.

6.2.2 Risk assessment

For all patients, the clinical nursing staff completed the CBO scale to assess the patients risk for pressure ulcer development (CBO, 2002). Currently, risk scales are seen

Study population	**Age** (mean±STD; range)	**Male** (n)	**Female** (n)
With NBE (n=5)	78±8; 66-86	2	3
Without NBE (n=31)	59±15; 21-86	14	17

Table 6.1: Composition of study population. NBE indicates non-blanchable erythema (i.e. grade I ulcer).

as the 'gold standard' risk assessment tests. In the CBO scale pressure ulcer development is based on factors, such as nutrition, mobility, mental state, sensory perception, incontinence, temperature, circulation, diabetes, age, and medication. A score below 8 indicates low risk, between 8 and 12 indicates medium risk, and above 12 indicates high risk for pressure ulcer development.

6.2.3 Sebutape recovery efficiency

A non-invasive method based on Perkins *et al.* (2001) was used to absorb cytokines and chemokines from the skin surface. For this method, Sebutapes (CuDerm Corporation, TX, USA), lipophilic polymeric silicone coated films (width=1.91 cm, length=2.86 cm), were gently applied to the skin surface. The recovery efficiency of Sebutapes for cytokines and chemokines was determined by spiking a known amount of IL-1α standard (200-LA; R&D systems, Abingdon, United Kingdom) onto the tapes and drying them under a stream of nitrogen. The tapes were stored at -80°C prior to analyzing the amount of IL-1α in the tape extracts (section 6.2.5 and 6.2.6). The results of this 'spiking' experiment are described in section 6.3.2.

6.2.4 Sebutape sample collection

The patients were asked not to use any skin care products the morning before tape sampling. Sebutapes were applied to the skin surface for a convenient period of 2 min, using blunt tweezers and gloved hands, to avoid cross contamination of skin proteins. Perkins *et al.* (2001) demonstrated that variations in sampling time between 1 min and 1 h did not influence the amount of IL-1α that was recovered from the skin surface for low sebum regions, such as the arm and lower back. The pressure used to press a Sebutape to the skin surface was kept constant by using a custom-built 'pressure-roller' (figure 6.1). This device consists of a small roller, which is attached to one end of a lever. The lever is connected to a spring, allowing the roller to be pressed down with a fairly constant pressure on each application. Moreover, all Sebutapes were applied by the same researcher.

Sebutapes were applied to various skin sites. For patients with grade I ulcers, these tapes were applied to three sites; immediately above the ulcer at the sacrum (S), at a 10 cm distance from the ulcer (10S), and on the volar aspect of the left forearm (A) (figure 6.2). The left arm was used as a normal appearing control site, since a

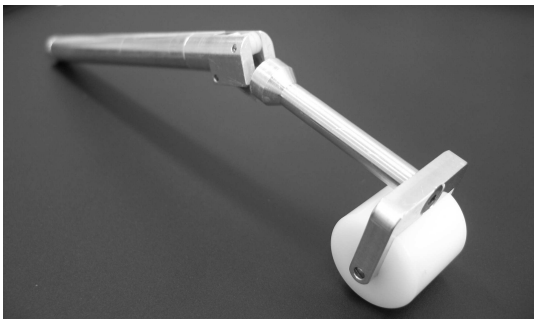


Figure 6.1: Image of the pressure-roller.

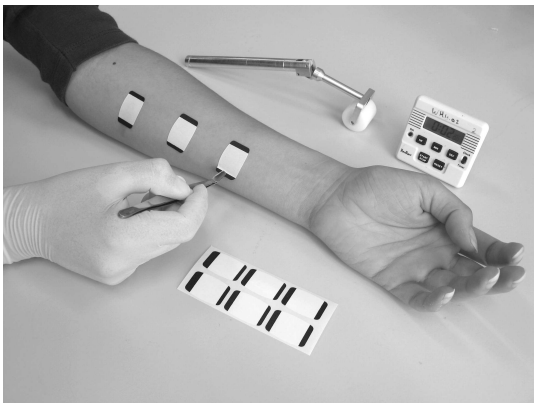


Figure 6.2: Sebutapes applied to the volar aspect of the left forearm.

pilot study on 5 healthy students demonstrated that the amount of IL-1α measured at the sacrum was comparable to the amount measured at the left arm. For patients without pressure ulcers, cytokines and chemokines were collected from the sacrum (S), as well as from the volar aspect of the left forearm (A) using Sebutape. All control sites (i.e. 10S and A) were confirmed to be intact, unblemished, and free of excessive hair by visual inspection prior to study initiation. For both patient groups, a tape was applied to the described skin site after unloading the sacrum for 30 min. The sacrum was unloaded prior to tape sampling to reduce the interference of the interface upon supine lying and sitting. After Sebutape sampling, the tapes were removed from the skin using blunt tweezers. Taking care not to crumble or fold the sample, each tape was placed flat into a vial (Sigma-Aldrich Co, St. Louis, Missouri, USA), with its adhesive side upward, and immediately frozen on ice. When the tapes of one patient were collected they were stored at -80°C until further processing (section 6.2.5).

6.2.5 Sebutape extraction

The Sebutape extraction process was based on a study by Perkins *et al.* (2001). Briefly, the tapes were thawed to room temperature and 2 ml of phosphate buffered saline (PBS; Sigma-Aldrich Co, St. Louis, Missouri, USA) solution was added to each vial. After immersion for 1 h, the tapes were sonicated for 10 minutes at 20°C, vortexed vigorously for 2 min, and additionally mixed with a pipette tip. The tape extracts were refrozen overnight at -80°C. The tape extracts were subsequently thawed, vortexed for 1 min and mixed with a pipette to recover the total extracts from the tapes. The amount of cytokine and chemokine (section 6.2.6) and total amount of protein (section 6.2.7) were determined in these tape extracts.

6.2.6 Cytokine and chemokine analyses

The levels of IL-1α, IL-1RA, and IL-8 were determined in the tape extracts by ELISA. For IL-1α, IL-1RA, and IL-8 (M9318, CLB, Amsterdam, the Netherlands) quantification, Maxisorp immunoplates (Nalge Nunc international, Roskilde, Denmark) were coated overnight at room temperature with capture antibody. Subsequently, the plates were blocked with PBS/0.5$\%$ BSA for 1 h at room temperature, after which the tape extracts and standards were added to the plate. After incubation for 1 h at room temperature and washing (PBS/ 0.005$\%$ Tween-20), the detection antibody was added. The plates were subsequently incubated with 1:10000 diluted streptavidin-HRP (M2032, Sanquin Reagents, Amsterdam, The Netherlands) for a period of 30 min. The enzyme reaction was initiated by adding 0.2 mg/ml of o-phenylenediamine dihydrochloride in 0.11 M acetate pH 5.5 and 0.03$\%$ hydrogen peroxide and stopped with 2M H_2SO_4. Dilution buffer (PBS/0.5% BSA/0.005% Tween-20) was used as a blank in this assay. Absorbance was determined at 490 nm using a plate reader. All antibodies and standards were purchased from R$\&$D systems (Abingdon, United Kingdom), unless otherwise indicated.

6.2.7 Total protein analyses

The QuantiPro BCA assay kit (QPBCA; Sigma-Aldrich Chemie B.V., Zwijndrecht, NL) was used to determine the total amount of protein in the tape extracts. The total amount of protein was assessed to correct for differences in protein loading during tape sampling. The QuantiPro working reagent was prepared by mixing 25 parts of reagent QA (a solution of sodium carbonate, sodium tartate, and sodium bicarbonate in 0.2 M NaOH, pH 11.25) with 25 parts of reagent QB (4% (w/v) bicinchoninic acid solution, pH 8.5). After reagents QA and QB were combined, 1 part of reagent QC (copper(II) sulfate) was added, and the working reagent was mixed until it was uniform in color. The BCA assay gives a linear response from 0.5 to 30 mg/ml of protein. All tape extracts, blanks (PBS), and standards were mixed in equal amounts with the working reagent and incubated for 1 h at 60°C. After cooling the reaction solution to room temperature, the absorbance was measured at 562 nm using a plate reader.

6.2.8 Statistics

The amount of cytokines and chemokines were normalized to the total amount of protein, to reduce the inter-subject variability in protein loading. The data were not assumed to be normal in distribution and, hence, were presented as box and whisker plots. Accordingly, the Wilcoxon Signed-Ranks test (SPSS 12.0.1, USA), was used to compare the normalized amounts of cytokines and chemokines at the sacrum (S), as well as at a 10 cm distance from the sacrum (10S), to that of the left arm (A). Differences were considered to be statistically significant at P-value$<$0.1.

6.3 Results

6.3.1 Risk assessment

The risk scores for patients with a grade I ulcer (i.e. non-blanchable erythema) at the sacrum were based on the CBO scale. Only three out of five patients with a grade I ulcer were actually classified as 'high risk' patients (score$>$12). One patient had a 'medium risk' for pressure ulcer development (score$=$10). Whereas, the other patient did not even seem to be at risk (score$=$5), although a grade I ulcer was clearly present at the sacrum. Based on the current data, 40% of the patients with a grade I ulcer would not have obtained adequate preventive measures in time. On the other hand, patients without pressure ulcers were all classified as 'low risk' patients (score$<$8).

6.3.2 Sebutape recovery efficiency

The Sebutape recovery efficiency was shown to be optimal for IL-1α (figure 6.3). Besides, the recovery efficiency seemed to be the same for each spiked concentration of IL-1α.

6.3.3 Sebutape sample collection

Figure 6.4 shows the normalized amount of IL-1α (pg/μg) for patients without pressure ulcers (a), as well as for patients with a grade I ulcer at the sacrum (b). Similar results were obtained when Sebutapes were applied 30 min before the current measurement. The corresponding values of IL-8 and IL-1RA were below the minimal detectable values of 12 and 31 pg/ml, respectively. A considerable variability was evident in the IL-1α values for each of the test conditions. The findings generally revealed higher levels of IL-1α associated with the sacrum (S) compared to those at the left arm (A). However, close examination of data for the five patients with a grade I ulcer, revealed this elevation to be localized over the ulcer site, with values at 10 cm away from the ulcer more comparable to those at the left arm (figure 6.4b). The high inter-subject variability was the most pronounced at the sacrum as can also be seen in figure 6.5, in which the normalized level of IL-1α at the left arm and the sacrum are plotted against the age of the patients. No correlation was found in the amount of IL-1α measured at the skin surface and the age of the patients, indicating

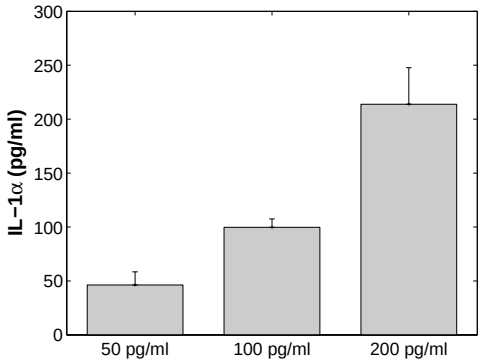
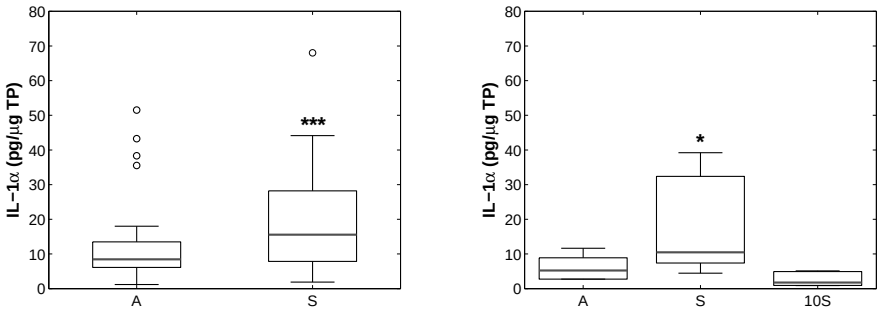


Figure 6.3: Sebutape recovery efficiency (mean+STD) upon spiking the tapes with various concentrations of IL-1α standard (50, 100 and 200 pg/ml).



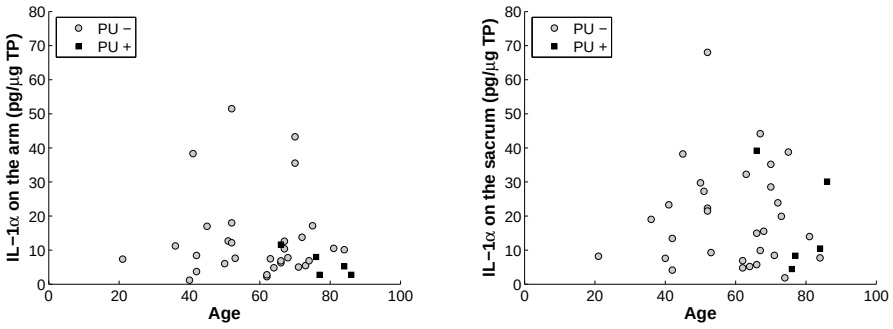
(a) (b)

Figure 6.4: Box-and-Whisker plots of IL-1α normalized to the total amount of protein (TP) at the left arm (A), the sacrum (S), and at a 10 cm distance from the sacrum (10S). The results are shown for patients without pressure ulcers (a), as well as for patients with a grade I ulcer at the sacrum (b). Outlayers are indicated by the sign $\circ$. * indicates P$<$0.1 and *** indicates P$<$0.01 compared to A.

the applicability of these measurements among different age groups. However, it can be observed that patients with a grade I ulcer were generally in the elder cohort of patients included in the study.

To further interrogate the magnitude of the IL-1α increase at the sacrum in comparison with the left arm, the ratio values are depicted in figure 6.6 for both patient groups. Superimposed on this figure is the ratio between the amount of IL-1α at the

sacrum and the left arm for five healthy young subjects (students), which were earlier assessed in a pilot study. For this young healthy group, their ratio values are approximately unity as depicted by the dashed line. This finding implies that the left arm might serve as a suitable control site. However, it should be noted that the age of the students (age range 26-32 years) differs substantially from that of the patient groups (age range 21-86 years). For patients without pressure ulcers, a small increase in IL-1α median ratio could be observed. For patients with a grade I ulcer at the sacrum, the relative increase in IL-1α median ratio is higher when compared to the patient group without pressure ulcers. More patients with a grade I ulcer should, however, be examined to compare both patients groups to one another by means of statistical analyses.



(a) (b)

Figure 6.5: IL-1α normalized to the total amount of protein (TP) at the left arm (a) and the sacrum (b) plotted against the age of patients without pressure ulcers (PU-) and patients with a grade I ulcer (PU+).

6.4 Discussion

The aim of the present study was to examine cytokines and chemokines, such as IL-1α, IL-1RA, and IL-8, in grade I ulcers *in vivo*. The Sebutape sampling method was used to collect cytokines and chemokines from the skin surface of patients. Patients with a grade I ulcer at the sacrum, as well as patients with no evidence of pressure ulcers participated in this study. For both patient groups, Sebutapes were applied to the sacrum as well as to the volar aspect the left forearm (i.e. normal appearing control site). In addition, for patients with a grade I ulcer a Sebutape was also applied to a normal appearing skin site at a 10 cm distance from the ulcer.

Currently, risk assessment scales are widely used to determine the patients risk for pressure ulcer development. Using the CBO scale only 60% of the five patients with a grade I ulcer were actually classified as 'high risk' patients in the present study.

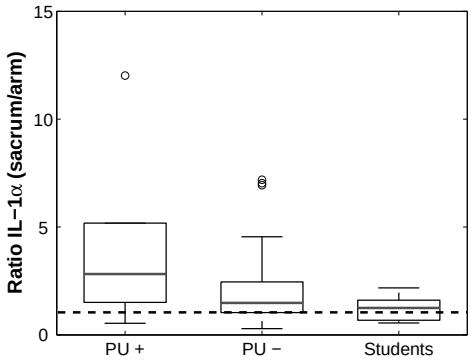


Figure 6.6: Box-and-Whisker plots of the ratio between the amount of IL-1α at the sacrum and the left arm for patients with a grade I ulcer (PU+), patients without pressure ulcers (PU-), and for students. Outlayers are indicated by the sign $\circ$. The dashed line equals a ratio of value 1.

This finding again emphasizes the need for improved pressure ulcer risk detection techniques.

The Sebutape recovery efficiency was shown to be optimal for IL-1α, since 100% of the 'spiked' IL-1α standards could be recovered from the tapes. Therefore, it seems that the Sebutape sampling method can effectively be used for assessing the skin surface on the presence of small proteins. Perkins *et al.* (2001) demonstrated that IL-1α and IL-1RA could both be detected on normal appearing skin sites of adult subjects, using the Sebutape sampling method. In the present study, a comparable basal level was found for IL-1α (pg/μg) at a representative control site (i.e. the volar aspect of the left forearm). However, no IL-1RA could be detected. This finding might, therefore, imply that our sampling method is not yet optimal for detecting other cytokines than IL-1α. On the other hand factors, such as the disease state of the patient or drug use (e.g. anti-inflammatory drugs), might influence the skin cytokine and chemokine levels. In addition, IL-8 could not be measured on normal appearing skin sites. This is not surprising, since IL-8 is known to be produced only on demand upon skin irritation or injury (Perkins *et al.*, 2001; Uchi *et al.*, 2000). However, the level of IL-8 on affected skin sites was below the minimal detectable value of 12 pg/ml in the present study. The recovery efficiency of the Sebutapes for IL-1RA and IL-8 will be evaluated in the near future. Dependent on the outcome of this study, adjustments may be made to the Sebutape sampling method. Furthermore, sustained pressures will be applied on the skin of healthy subjects to determine which cytokines and chemokines can actually be detected at the skin surface *in vivo* upon mechanical loading. Earlier *in vitro* studies by our group, however, demonstrated increased levels of IL-1α, IL-1RA, and IL-8 upon mechanical loading of an epidermal model system (chapter 3 and 4).

Perkins *et al.* (2001) demonstrated elevated levels of IL-1α and IL-1RA in infants with diaper rash compared to uninvolved clinically normal skin sites. In the present study, an increase in the level of IL-1α was found at the sacrum compared to the control site (i.e. the volar aspect of the left forearm). This increase was observed for both patient groups and might be caused by sustained mechanical loading of the sacrum upon supine lying and sitting during hospital stay. By contrast, there were minimal differences between the levels of IL-1α at either skin site for young healthy subjects. To determine whether this local difference in IL-1α is age dependent or caused by prolonged hospital stay, healthy elderly subjects should be assessed as well. Nevertheless, the relative increase (ratio) in IL-1α between the sacrum and the left arm seemed somewhat higher for patients with a grade I ulcer compared to patients without pressure ulcers. These preliminary data are promising and might imply that a threshold level is present for IL-1α beyond which visible tissue damage, as evidenced by non-blanchable erythema, occurs. Indeed such a threshold was observed earlier in *in vitro* studies, as reported in chapter 3 and 4. Although, it has to be noted that the high IL-1α median ratio for patients with a grade I pressure ulcer might also be caused by lower absolute amounts of IL-1α at the left arm, as opposed to higher absolute amounts at the sacrum. More patients with a grade I ulcer should be examined to validate the hypothesis of such a threshold.

The IL-1α levels were only locally raised, since at a 10 cm distance from the ulcer the level of IL-1α was almost comparable to that of the left arm. This finding might indicate that grade I ulcers give a local tissue reaction, that can probably not be measured systemically. Furthermore, the level of IL-1α strongly varied among patients, whereas, the total protein levels remained remarkably consistent (data not shown). This inter-subject variability in IL-1α increased even more on affected skin sites, such as the sacrum. A high variability in IL-1α was also observed in earlier *in vitro* studies in which epidermal tissue was irritated in a mechanical way (chapter 3 and 4). Due to this high variability in IL-1α, the affected skin site should always be compared to a control site in the same patient.

In conclusion, the findings of the present study suggest a threshold level for IL-1α beyond which visible tissue damage, such as a grade I ulcer, might occur. More patients with a grade I ulcer will be examined in the near future to validate the hypothesis of such a threshold. IL-1α might not be appropriate as a single parameter to indicate the damage state of skin tissue. Due to the high inter-subject variability in IL-1α, a combination of markers, rather than a single marker, might be more informative about the tissue status. Therefore, the relation with other biochemical markers (i.e. lactate, urea, purines) or even physiological indicators (i.e. skin redness, trans epidermal water loss (TEWL), transcutaneous oxygen ($tcPO_2$) and carbon dioxide ($tcPCO_2$), blood flow, and blood content and oxygenation) for mechanically-induced tissue damage should be investigated (Bader, 1990; Bader *et al.*, 2005; Clarys *et al.*, 2000; Ek *et al.*, 1984; Hagisawa *et al.*, 1994; Knight *et al.*, 2001; Nuutinen *et al.*, 2003; Polliack *et al.*, 1997). A non-invasive method for pressure ulcer risk assessment is of utmost importance, particularly in clinical situations. If biosensor technology were available, a multi-factor based biosensor might eventually be developed for measuring and monitoring typical ulcer locations, such as the sacrum, trochanter, heels, and

ischial tuberosities, and determine the patients risk for pressure ulcer development.

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Chapter 7

General discussion

7.1 Introductory remarks

Pressure ulcers are areas of soft tissue breakdown resulting from sustained mechanical loading of the skin and underlying tissues (APUAP, 1998). The prevalence of pressure ulcers is unacceptably high and varies from 15% in general hospitals to 24% in nursing homes (Halfens *et al.*, 2006). In our view, this high prevalence is partly due to limited risk assessment tools. Currently, risk assessment is mainly performed by questionnaires or scales, which are solely based on expert opinion or literature review and have a limited scientific background (Schoonhoven *et al.*, 2002). These risk assessment scales do not predict pressure ulcer development satisfactorily, since up to 30% of the hospitalized patients with pressure ulcers are still misclassified (Schoonhoven *et al.*, 2002). A first step in developing a new risk assessment tool should be focused on early detection of these ulcers.

The present thesis focuses on biochemical markers for early detection of superficial pressure ulcers. An *in vitro* model of the epidermis, the most superficial layer of the skin, was used to evaluate biochemical markers upon mechanical loading. Specific pressures were applied on top of these epidermal equivalents using indenters and additional weights, in a custom-built loading device. It was hypothesized that pressure ulcers are preceded by an inflammatory response. Therefore inflammatory mediators, in particular specific cytokines and chemokines, were evaluated for objective detection of mechanically-induced epidermal damage. To determine whether cytokines and chemokines are suitable early markers, their release profiles were studied for various load magnitudes and durations. Previous studies demonstrated an inverse relationship between tissue damage and external load magnitude and duration (Breuls *et al.*, 2003; Daniel *et al.*, 1981; Kosiak, 1959; Reswick and Rogers, 1976). Thus, both a large load magnitude applied for a short period of time and a smaller load applied for a longer time period are known to result in tissue damage. Furthermore, cytokines were used to study the role of urine on the susceptibility of the epidermis to mechanical loading. Urinary incontinence is widely recognized as an important risk factor for pressure ulcer formation, although scientific proof was still lacking. In addition, a clinical study was performed to determine whether the identified biochemical markers can actually be measured in a clinical setting using an existing sampling method (Perkins *et al.*, 2001).

In the following sections the used methodology is discussed namely: the epidermal equivalent (section 7.2), the custom-built loading device (section 7.3), biochemical markers (section 7.4), and the *in vivo* marker collection method (section 7.5). The main results of the *in vitro* and *in vivo* studies are summarized in section 7.6. Next, directions for future research are indicated (section 7.7).

7.2 Epidermal equivalent

Commercially available EpiDerm cultures (MatTek Corporation, Ashland, MA, USA) were used as an *in vitro* model of the epidermis. Such a model enables improved control of experimental conditions (i.e. temperature, humidity, CO_2), and offers the

potential of performing well-controlled experiments (i.e. mechanical loading experiments). To date, EpiDerm cultures have mostly been employed as diagnostic models for *in vitro* toxicology testing as an alternative to whole animal testing (Bernhofer *et al.*, 1999; Coquette *et al.*, 2003; Perkins *et al.*, 1999; Faller and Bracher, 2002; Faller *et al.*, 2002). However, there are several arguments indicating that EpiDerm cultures can also be used to study the effects of mechanical loading (chapter 3). First, the general morphology of the EpiDerm cultures is comparable to that of normal human epidermis (Netzlaff *et al.*, 2005). All epidermal strata can be distinguished in these cultures (i.e stratum basale, stratum spinosum, stratum granulosum, and stratum corneum) (chapter 1). Besides, the stratum corneum, which forms the skin barrier and protects the skin against excessive water loss, the ingress of foreign chemicals and micro-organisms, UV irradiation, and mechanical insults, is well-developed. The stratum corneum of these EpiDerm cultures exhibit a normal basket-weave pattern and the extrusion of the lamellar body contents at the interface between the stratum granulosum and stratum corneum is complete (Ponec *et al.*, 2002). Second, lipid analysis, further, revealed the presence of all major lipid classes in the EpiDerm cultures (Ponec *et al.*, 2002). The expression and location of differentiation markers was also similar to that of normal human epidermis (Ponec *et al.*, 2002; Netzlaff *et al.*, 2005). Third, the mechanical properties of the EpiDerm cultures seem comparable to the properties of skin measured *in vivo* by Hendriks (2005) (unpublished results). However, due to the small dimensions of these cultures (diameter=8 mm) it was difficult to perform uniaxial tensile tests. Therefore, the mechanical properties were measured using spherical indentation (Cox *et al.*, 2006). To date, very few EpiDerm cultures have been mechanically tested. To get a better indication of the mechanical properties, different batches of EpiDerm cultures should be tested and compared to *ex vivo* human epidermis.

Biochemical markers originating from keratinocytes in the most superficial layer of the skin, the epidermis, might be suitable for non-invasive pressure ulcer risk detection. Therefore, the human epidermal equivalent (EpiDerm) was employed in the current thesis to evaluate biochemical markers upon mechanical loading. However, processes such as damage progression or tissue repair cannot be studied using an epidermal model system alone, mainly due to the lack of fibroblasts. These cells are known to interact with keratinocytes and influence epidermal regeneration (Ghalbzouri *et al.*, 2002). A full-thickness skin model (i.e. epidermis and dermis) or *in vivo* measurements would, therefore, be more appropriate to study these damage and/or repair processes (Bronneberg and Bouten, 2005).

Pressure ulcers mostly occur in elderly and/or diseased subjects (e.g. diabetes). Although, EpiDerm cultures are currently composed of cells from healthy adult subjects. It might, therefore, be of interest to determine the effect of mechanical loading on epidermal equivalents composed of cells from elderly or diseased subjects.

Problems that were encountered using EpiDerm cultures were a high inter- and intra-batch variability in biochemical markers (i.e. cytokines and chemokines) (chapter 2 and 3). This variability was also observed in other studies using EpiDerm cultures (Faller and Bracher, 2002). Furthermore, various transport-related problems were encountered. EpiDerm cultures are shipped overseas on medium-supplemented

agarose gels together with freezing-elements. Upon customs delay, the supplemented medium might, however, not be sufficient. In addition, the cultures might become to warm (>4°C). These factors affect the viability of the EpiDerm cultures. Besides, the best reproducibility was known to be obtained if the EpiDerm cultures were used consistently on the same day.

7.3 Custom-built loading device

In the present thesis clinically relevant pressures were applied using indenters (diameter=5 mm) and additional weights in a custom-built loading device (chapter 2). Various loading regimes (i.e. load magnitudes and durations) can easily be applied to EpiDerm cultures and reproducible results can be obtained (chapter 2-5). An *in vitro* model for pressure ulcer development was used in the current thesis, since it is not practical and ethical to induce various degrees of tissue damage in human subjects.

The current *in vitro* model system has proven successful to study the effect of a pressure ulcer risk factor, such as urine, on the susceptibility of the epidermis to mechanical loading (chapter 5). Therefore, this model system might also be useful for industry to determine the effect of ointments, such as Sanyrene (Laboratoires Urgo, France), for pressure ulcer prevention.

Due to the geometrical constraints of the EpiDerm cultures, an inhomogeneous strain field is currently induced upon loading. In figure 7.1, it can be observed that the EpiDerm cultures (diameter=8 mm) are situated 1 mm above a rigid culture plate, since they are located on a membrane of a cell culture insert. When a pressure is applied to the center of these EpiDerm cultures, there will be bending of the epidermal tissue. Therefore, it is expected that both shear and compressive strains are apparent in the epidermal tissue at the edges and underneath the indenter, respectively. Currently, an axi-symmetric finite element model is being developed to determine a detailed strain distribution in the EpiDerm cultures during mechanical loading. Previous studies by our group already demonstrated that sustained cell and

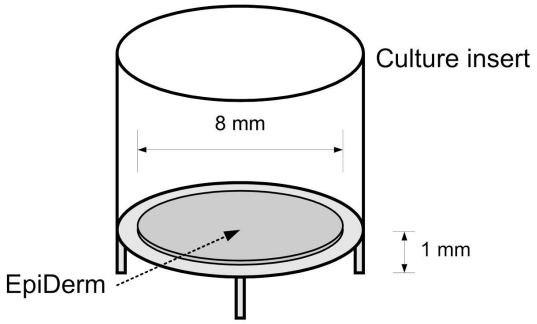

Figure 7.1: Schematic representation of the location of an EpiDerm culture inside a cell culture insert.

tissue straining can actually result in cell and tissue breakdown (Bouten *et al.*, 2001; Breuls *et al.*, 2003; Gawlitta *et al.*, 2007; Peeters *et al.*, 2005). A threshold level for tissue damage might, therefore, be formulated based on the strains within the epidermal tissue. To validate such a finite element model, the displacement of the indenter should be measured in future experiments, for instance by means of a linear variable displacement transducer.

A different approach might be to simplify the current strain field. Homogenous tissue deformation can, for instance, be obtained by placing porous filters beneath the EpiDerm cultures during mechanical loading. The pore size of such a filter should be large enough to allow transport of biochemical markers and small enough to prevent tissue bending. However, the first results using a porous filter were not that promising, since the biochemical markers all adhered to the glass filter material. Other filter materials should, therefore, be evaluated.

It might also be of interest to determine the specific effect of either shear stress or friction on the EpiDerm cultures. Since, both factors are known to contribute to pressure ulcer development (CBO, 2002). However, in comparison with pressure application, it is much more difficult to apply these loading modes in an *in vitro* setting due to the small dimensions of the EpiDerm cultures. Furthermore, shear stress is thought to cause maceration and detachment of superficial skin layers (i.e. epidermis) *in vivo* (Dinsdale, 1974; Reuler and Cooney, 1981). A full-thickness skin model might, therefore, again be more appropriate to use.

7.4 Biochemical markers

Goldstein and Sanders (1998) demonstrated that superficial pressure ulcers (grade I ulcers) in pigs showed morphological signs of inflammation. It is therefore hypothesized that pressure ulcers are preceded by an inflammatory response. Inflammatory mediators (i.e. cytokines and chemokines) were evaluated as biochemical markers for early and objective detection of mechanically-induced epidermal damage, such as pressure ulcers. Various cytokines and chemokines (i.e. interleukin 1 α (IL-1α), IL 1 receptor antagonist (IL-1RA), interleukin 8 (CXCL8/IL-8), tumor necrosis factor α (TNF-α), macrophage inflammatory protein 3 α (CCL20/MIP-3α), monocyte chemotactic protein 1 (CCL2/ MCP-1), and growth-regulated protein precursor α (CXCL1/GRO-α)) were studied in the present thesis (chapter 3 and 4). These cytokines and chemokines all originate from keratinocytes in the epidermis and might, therefore, be detected on the skin surface upon skin irritation or injury as a result of mechanical loading. Until now, they were mainly used as screening parameters for measuring skin reactivity to various chemicals, as well as UV irradiation (Bernhofer *et al.*, 1999; Faller and Bracher, 2002; Flint *et al.*, 1998; Nickoloff and Naidu, 1994; Oxholm *et al.*, 1988).

Cytokines and chemokines were studied in an *in vitro* model of the epidermis (i.e. EpiDerm culture). No fibroblasts were incorporated into this model. Therefore, the release pattern of these cytokines and chemokines could conceivable be different in the *in vivo* condition. Since, fibroblasts are known to interact with keratinocytes in

the epidermis (Ghalbzouri *et al.*, 2002, 2004). The initial release of IL-1α and IL-1RA from the epidermis is not expected to be changed by the interference of fibroblasts. Since, IL-1α and IL-1RA were found to be stored intracellularly (chapter 4) and are known to be exclusively released from keratinocytes upon cell membrane disruption (Uchi *et al.*, 2000). However, for cytokines and chemokines that are not constitutively produced (e.g. IL-8), it might also be of interest to study their release in a full-thickness skin equivalent or in an epidermal equivalent cultured with medium derived from fibroblasts.

Various factors might inflict with the use of cytokines and chemokines for pressure ulcer detection and risk assessment. First, the disease state (e.g. cancer) or drug use (e.g. anti-inflammatory drugs) of patients might interfere with the regulation of these biochemical markers in skin tissue (Perkins *et al.*, 2001). Second, deep tissue injury (i.e. pressure ulcers that initiate in deep tissue layers) might not be detected using these markers. Therefore, muscle specific markers (i.e. creatine kinase, myosin heavy chain, fatty acid binding protein, myoglobin, and skeletal troponin I) should be measured as well in either blood or urine samples (Sorichter *et al.*, 1997, 1998).

7.5 *In vivo* marker collection

Perkins *et al.* (2001) developed a non-invasive sampling method to absorb cytokines and chemokines from the skin surface using Sebutape. Sebutape is a lipophilic polymeric silicone coated film, which has been used by many investigators in clinical studies for the extraction of sebum from the skin (Pagnoni *et al.*, 1994). These tapes cause minimal stripping of corneocytes or damage to the stratum corneum, and inflict minimal pain to the patient. Furthermore, this technique provides a cheap method for collecting cytokines and chemokines from the skin surface. Perkins *et al.* (2001) demonstrated that IL-1α, IL-1RA, and IL-8 could all be detected using the Sebutape sampling method. However, in the present thesis IL-1α could only be measured. This might indicate that our sampling method is not yet optimal for detecting other cytokines or chemokines than IL-1α. The disease state or drug use of the patients that participated in this study might also interfere with the skin cytokine or chemokine levels. These factors should, therefore, be registered in future studies.

Currently, only the sacral area, which is the most common site for pressure ulcer occurrence (Meehan, 1994), was examined using the Sebutape sampling method (chapter 6). Grade I ulcers at other typical ulcer sites, such as the trochanteric and ischial areas, were not observed upon performing this clinical study. On the other hand, grade I ulcers at the heels were found, but these ulcers could not be assessed using the Sebutape sampling method. The Sebutapes did not properly adhere to heels due to their curved surfaces. The same problem was encountered when the skin was extremely wrinkled. The sampling method should be improved, so that it can be used on all different skin surfaces. Furthermore, an ulcer cannot be optimally assessed when its size was significantly smaller than the size of the Sebutape. This limitation should also be taken into account, when designing an improved sampling method.

7.6 Main results

Various cytokines and chemokines (i.e. IL-1α, IL-1RA, TNF-α, IL-8, MIP-3α, MCP-1, and GRO-α) were evaluated as biochemical markers for early detection of superficial pressure ulcers. In chapter 3 increasing load magnitudes (0, 50, 75, 100, 150, and 200 mmHg) were applied to EpiDerm cultures *in vitro* for 24 h, to cause various degrees of epidermal damage. IL-1α, IL-1RA, IL-8, and TNF-α were shown to be released as a result of sustained mechanical loading. A first increase in the release of these biochemical markers was observed at the relatively low pressure of 75 mmHg, when structural tissue damage was barely visible. This important finding indicates the sensitivity of the epidermis for these markers at relatively low pressure levels. Furthermore, a threshold seems present for these markers, beyond which visible tissue damage occurs. MIP-3α, MCP-1, and GRO-α could, however, not be measured. These chemokines were either not released after mechanical loading or could simply not be detected using the current immunoassay technique. In chapter 4, EpiDerm cultures were subjected to either 0 or 150 mmHg in a custom-built loading device for various load durations (1, 2, 4, 6, 8, 16, and 24 h). After 1 h of epidermal loading and before the onset of visible tissue damage, a first increase in the release of IL-1α, IL-1RA and IL-8 could be observed. Therefore, these markers are suitable for early detection of mechanically-induced epidermal damage in an *in vitro* setting. On the other hand, TNF-α seemed less fit for early damage detection, since this marker was only raised when the first signs of epidermal damage were already apparent. Furthermore, the amount of TNF-α was very small and could hardly be detected using a 'high sensitive' immunoassay technique. From the *in vitro* studies it can be concluded that IL-1α, IL-1RA, and IL-8 are promising biochemical markers for early detection of superficial pressure ulcers.

In chapter 5, biochemical markers, such as IL-1α and IL-1RA, were used to study the effect of synthetic-urine (s-urine) on the susceptibility of the epidermis to mechanical loading. An increase in epidermal damage, as well as in the release of IL-1α and IL-1RA was observed when both s-urine and pressure were applied. Although these findings are obtained *in vitro*, they might imply that urinary incontinent patients have a higher risk of developing pressure ulcers. Therefore, this study justifies the use of urinary incontinence as a risk factor for pressure ulcers.

As proof of principle, a clinical study was performed to determine whether biochemical markers, such as IL-1α, IL-1RA and IL-8, could also be detected in an *in vivo* setting. The Sebutape sampling method was used to collect cytokines and chemokines from the skin surface in a non-invasive way (Perkins *et al.*, 2001). Patients with a grade I ulcer (i.e. non-blanchable erythema) at the sacrum, as well as patients without pressure ulcers participated in this study. For both patient groups, Sebutapes were applied to the sacrum as well as to the volar aspect of the left forearm (i.e. control site). Currently, only IL-1α could be detected using the Sebutape sampling method. For both patient groups, an increase in the absolute level of this cytokine was found at the sacrum compared to the left arm. This increase in IL-1α might be caused by sustained mechanical loading of the sacrum upon supine lying and sitting and was observed in spite of a high inter-subject variability in IL-1α. For patients

with a grade I ulcer at the sacrum, the relative increase in IL-1α median ratio is higher when compared to the patient group without pressure ulcers. This result is promising and might indeed imply that a threshold is present for IL-1α beyond which visible tissue damage, as evidenced by non-blanchable erythema, occurs. More patients with a grade I ulcer will be examined in the near future to validate the hypothesis of such a threshold. IL-1α might, however, not be used on its own to indicate the damage state of skin tissue. Due to the high inter-subject variability in IL-1α, a combination of markers, rather than a single marker, might be more informative about the tissue status. A non-invasive method for early detection of superficial pressure ulcers is of utmost importance, particularly in clinical situations.

7.7 Directions for future research

The specific role of inflammatory mediators in mechanically-induced epidermal damage, such as pressure ulcers, needs to be fully unraveled in future experiments. The present thesis demonstrates a rapid increase in cytokines and chemokines, such as IL-1α, IL-1RA, and IL-8, upon mechanical loading. Furthermore, a threshold level was found *in vitro* beyond which visible tissue damage occurred. However, the physiological implications of an increase in cytokines and chemokines upon mechanical loading is not yet clear. Do these markers mainly function as inflammatory mediators, initiating a cascade that will (normally) lead to tissue repair? And is the extent of an increase merely indicative of the amount of tissue damage? Or is there a single marker, or a combination of markers that are raised upon mechanical loading in some subjects, but not in others? Or is the release rate of these markers perhaps altered in subjects that are highly susceptible to mechanically-induced skin damage? Many questions need to be addressed before these biochemical markers can actually be used for early detection of pressure ulcers.

Future *in vitro* studies should focus on the regulation of cytokines and chemokines in more detail. Reswick and Rogers (1976) demonstrated that certain combinations of load magnitude and duration will lead to tissue breakdown in humans. Cytokines and chemokines were evaluated as early damage markers for various combinations of load magnitude and duration. However, the number of combinations was far from comprehensive. Second, a finite element model should be developed to determine the strains within the epidermal tissue upon mechanical loading. Since, interface pressures are not really indicative of the mechanical conditions within the epidermal tissue. This approach might reveal a relationship between the level of tissue strains and cytokine and chemokine release, as well as a threshold for tissue damage. Third, various risk factors for pressure ulcer development might be examined *in vitro*. The effect of urinary incontinence was already studied. However, it might also be of interest to investigate the effect of factors, involving nutrition, skin temperature, and medication (CBO, 2002), on the susceptibility of the epidermis to mechanical loading. In addition, factors such as high age and disease (e.g. diabetes) might be studied by employing epidermal equivalents that are composed of cells from either elderly or diseased subjects. *Ex vivo* epidermis obtained from elderly or diseased subjects might

be used as well. Fourth, a full-thickness skin equivalent (i.e. epidermis and dermis) might be used to study the interaction of keratinocytes and fibroblasts upon mechanical loading. Due to the presence of fibroblasts, the release rate and/or amount of cytokines and chemokines might be somewhat altered. In addition, tissue repair might be investigated after load removal.

The clinical study that is described in the current thesis, will be extended in the near future. More patients with a grade I ulcer (i.e. non-blanchable erythema) will be examined, using the Sebutape sampling method for non-invasive assessment of skin cytokines and chemokines. A high inter-subject variability was observed for IL-1α in the present and other related studies (Perkins *et al.*, 2001). To identify changes in skin tissue as a result of non-blanchable erythema, it is essential to collect IL-1α simultaneously from a normal appearing skin site (i.e. at a 10 cm distance from the ulcer and the volar aspect of the left forearm) in the same patient. In this way, it can be determined whether there is a threshold value for IL-1α beyond which visible tissue damage (i.e. non-blanchable erythema) occurs. The current study might, however, benefit from some changes. First, the Sebutape sample collection method should be improved, to be able to measure other cytokines and chemokines than IL-1α. If possible, curved skin sites (i.e. heels) should also be studied in future experiments using the improved sampling method. Second, the basal levels of cytokines and chemokines should be measured on healthy elderly subjects at different skin sites. In this way, suitable control sites can be determined for typical ulcer locations, such as the sacrum and the heels. Until now, local differences in IL-1α were only studied in healthy subjects of a much younger age than the patients that participated in the clinical study. For these healthy young subjects no differences were observed between the sacrum and the volar aspect of the left forearm. Third, it might be of interest to perform longitudinal measurements on patients to determine the regulation of cytokines and chemokines in time. Furthermore, follow-up studies should be performed to investigate whether patients will eventually develop pressure ulcers if their cytokines and chemokine levels are raised above a certain level.

In vivo studies on healthy subjects should be performed as well. Using a uni-axial loading device (Knight *et al.*, 2001), sustained pressures should be applied on the skin surface without inflicting pain. In this way, the relation between *in vivo* skin loading and the regulation of cytokines and chemokines can be evaluated. The relation between cytokines and chemokines and other biochemical markers for skin loading, such as lactate, urea, and purines, should be investigated as well (Bader *et al.*, 2005; Knight *et al.*, 2001; Polliack *et al.*, 1997). These biochemical markers can be obtained from sweat metabolites. Last, a relation between biochemical markers and physiological indicators for mechanically-induced tissue damage (i.e. skin redness, trans epidermal water loss (TEWL), transcutaneous oxygen ($tcPO_2$) and carbon dioxide ($tcPCO_2$), blood flow, and blood content and oxygenation) should be obtained (Bader, 1990; Clarys *et al.*, 2000; Ek *et al.*, 1984; Hagisawa *et al.*, 1994; Nuutinen *et al.*, 2003).

A non-invasive method for measuring and monitoring tissue status is of utmost importance, particularly in clinical situations. Cytokines and chemokines seem promising markers for early detection of superficial pressure ulcers. A combination of mar-

kers, rather than a single marker, is however required to sufficiently monitor the status of skin tissue. Currently, the field of biosensor technology is emerging (Aytur *et al.*, 2006; Pejcic *et al.*, 2006). A biosensor might eventually be developed that is able to monitor various biochemical and physiological markers to determine the patients risk for pressure ulcer development. Such a biosensor should be accurate, safe, inexpensive, compact, easy-to-use, and allow rapid and real-time tissue monitoring.

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Samenvatting

Doorliggen of te wel decubitus is een beschadiging van zachte weefsels veroorzaakt door aanhoudende drukbelasting. De gevolgen van decubitus zijn bijzonder vervelend. De wonden zijn veelal pijnlijk en moeilijk te behandelen en kunnen het verblijf van een patient in een ziekenhuis verlengen. Decubitus is daarom een serieus probleem voor de gezondheidszorg. De prevalentie van decubitus is nog steeds hoog in Nederland. Afhankelijk van de aard van het instituut waar de meting gedaan is, varieert deze tussen 10 en 24%. De hoge prevalentie cijfers zijn veelal te wijten aan een beperkte risicoanalyse methode. Op dit moment wordt er gebruik gemaakt van risicoscorelijsten zoals de Norton, Braden en Waterlow. Deze lijsten hebben een zwakke wetenschappelijke onderbouwing en voorspellen de gevoeligheid van een patient nog niet nauwkeurig genoeg. Hierdoor kan het voorkomen dat risico-patiënten niet op tijd preventieve maatregelen ontvangen om het ontstaan van decubitus tegen te gaan.

De Landelijke Prevalentiemeting Decubitus heeft aangetoond dat graad I decubitus, het eerste stadium van oppervlakkige wonden aan de huid, in 50% van de gevallen voorkomt. Graad I decubitus wordt geclassificeerd als 'niet-wegdrukbare roodheid'. Op dit moment wordt er gebruik gemaakt van een doorzichtig plaatje om graad I decubitus te detecteren. Het doorzichtige plaatje wordt hierbij op de rode huid gedrukt. Indien de huid onder het plaatje rood blijft is er sprake van graad I decubitus. De beschreven techniek detecteert echter de gevolgen van weefselschade aan de huid (ontsteking). Terwijl juist het ontstaan van decubitus wonden voorkomen dient te worden. Verbeterde risicoanalyse methodes zijn dan ook noodzakelijk. Het onderzoek beschreven in dit proefschrift richt zich op cytokines en chemokines (ontstekingsmediatoren) voor de vroege detectie van oppervlakkige decubitus. De detectie van weefselreacties voorafgaand aan graad I decubitus is een mogelijke eerste stap in het ontwikkelen van een nieuwe risicoanalyse methode.

Er is een belastingsapparaat ontwikkeld om de schadelijke effecten van mechanische belasting te bestuderen in een *in vitro* model van de epidermis, de bovenste laag van de huid. De commercieel-verkrijgbare EpiDerm samples zijn hierbij gebruikt als modelsysteem, aangezien hun morfologische eigenschappen vergelijkbaar zijn met die van humane epidermis. Verschillende gradaties van epidermal schade werden verkregen door toenemende belastingen en belastingstijden op te leggen.

Gebruikmakend van het beschreven belastingsapparaat zijn verschillende cytokines en chemokines (IL-1α, IL-1RA, TNF-α, CXCL8/IL-8, CCL20/MIP-3α, CCL2/MCP-1

en CXCL1/GRO-α) gevalueerd als biochemische markers voor mechanisch-geïnduceerde epidermale schade. Alleen, IL-1α, IL-1RA, IL-8 en TNF-α werden uitgescheiden onder mechanische belasting. Het secretie patroon van deze cytokines en chemokines werd bestudeerd bij verschillende belastingen en belastingstijden. Na 24 uur belasten met drukken variërend tussen 0 en 200 mmHg werd een eerste toename in de secretie van IL-1α, IL-1RA, IL-8 en TNF-α waargenomen bij een druk van 75 mmHg. Terwijl er nauwelijks structurele epidermale schade zichtbaar was. Er blijkt dus een drempelwaarde aanwezig te zijn voor deze biochemische markers waarboven structurele epidermale schade optreedt. Bovendien werd er na 1 uur belasten met 150 mmHg een toename in IL-1α, IL-1RA en IL-8 waargenomen. Deze markers lijken daarom uitermate geschikt voor de vroege detectie van mechanisch-geïnduceerde epidermale schade in een *in vitro* setting. TNF-α lijkt minder geschikt voor de vroege detectie van schade. Aangezien een toename in TNF-α pas werd waargenomen op het moment dat de eerste tekenen van schade al zichtbaar waren. Bovendien was de hoeveelheid van TNF-α zodanig laag, dat het nauwelijks gedetecteerd kon worden.

Urine incontinentie wordt wereldwijd gezien als een belangrijke risico factor voor decubitus, terwijl het wetenschappelijk bewijs hiervoor ontbreekt. Biochemische markers, zoals IL-1α and IL-1RA, zijn daarom gebruikt om het effect van synthetische urine op de gevoeligheid van de epidermis voor mechanische belasting te bestuderen. Zodra zowel synthetische urine als druk werd aangebracht, werd er meer structurele epidermale schade en een toename in IL-1α en IL-1RA waargenomen. Deze resultaten impliceren dat urine incontinente mensen eerder decubitus kunnen ontwikkelen.

Om te bepalen of IL-1α, IL-1RA en IL-8 daadwerkelijk gedetecteerd konden worden in een *in vivo* setting, is er een klinische studie uitgevoerd. Hierbij is gebruik gemaakt van Sebutape, een zelfklevend folie, om de biochemische markers op een niet-invasieve en pijnloze manier van de huid op te nemen. Patiënten met graad I decubitus op hun sacrum en patiënten zonder decubitus namen deel aan deze studie. Voor beide patiënt-groepen werden Sebutapes op het sacrum en op de binnenzijde van de linker onderarm (controle positie) aangebracht. Op dit moment kon alleen IL-1α gedetecteerd worden aan de hand van de Sebutape methode. Voor beide patiëntgroepen werd een toename in de absolute hoeveelheid van IL-1α waargenomen op het sacrum ten opzichte van de linker arm. Deze toename in IL-1α kan veroorzaakt zijn door aanhoudende mechanische belasting van het sacrum tijdens het langdurig zitten en liggen in bed tijdens het verblijf in het ziekenhuis. Voor patiënten met graad I decubitus op het sacrum, werd echter een relatieve toename waargenomen in de mediaan ratio waarde van IL-1α ten opzichte van patiënten zonder decubitus. Dit resultaat is veelbelovend en impliceert wederom dat er een drempelwaarde is voor IL-1α waarboven zichtbare weefselschade, zoals niet-wegdrukbare roodheid, optreedt.

Cytokines en chemokines lijken veelbelovende biochemische markers voor de vroege detectie van oppervlakkige decubitus. Een combinatie van verschillende type markers lijkt echter nodig te zijn, in plaats van één specifieke marker, om de toestand van zachte weefsels, zoals huid, te beschrijven. Op dit moment is de biosensor technologie in opkomst. Uiteindelijk zou er een niet-invasieve biosensor ontwikkeld kunnen worden, voor verschillende biochemische en fysiologische markers, die het risico van de patiënt op het ontwikkelen van decubitus zou kunnen aantonen.

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Debbie Bronneberg,
Eindhoven, Augustus 2007

Curriculum Vitae

Debbie Bronneberg is geboren op 13 juli 1978 in Heerlen. In 1996 behaalde zij haar VWO diploma aan het Eykhagen College in Landgraaf. Aansluitend studeerde zij een jaar Werktuigbouwkunde aan de Technische Universiteit Eindhoven. Na het behalen van haar propedeuse besloot zij over te stappen naar de studie Biomedische Technologie aan de Technische Universiteit Eindhoven. Als onderdeel van deze studie liep zij stage aan Georgia Institute of Technology in Atlanta (USA), waar zij onderzoek deed naar de oriëntatie van endotheelcellen onder schuifspanning. Haar afstudeerwerk richtte zich op de regulatie van matrix metalloproteïnases in een modelsysteem van een bloedvat. In 2003 besloot zij promotieonderzoek te doen aan de faculteit Biomedische Technologie van de Technische Universiteit Eindhoven, resulterend in dit proefschrift. Dit project maakte deel uit van het decubitus onderzoek binnen de groep Biomechanica en Tissue Engineering. Vanaf 1 september 2007 is zij als Research scientist verbonden aan Philips Research.