Host defense peptides' effects on wound healing

Bachelor thesis in Medical and Molecular Biology

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Preface

This study is a 6th semester bachelor project written in molecular and medical biology. It is part of the Natural Science program at Roskilde University in Denmark. The project is written as a scientific literature report containing an experimental part supported by a theoretical background. The topic “Host defense peptides’ effect on wound healing” was chosen due to our interest in the field of medical biology and because host defense peptides are promising candidates in advancing wound healing. The project is written by Dunia Shagiwal, Julia Poniewierska, Laura Niclasen and Redaa Al-Makdisi Razeeghi in the spring semester of 2016. We would like to express our gratitude to supervisor Michelle Vang for her guidance and supervision throughout this study.

Abstract

Host defense peptides (HDPs) are molecules with defense mechanisms against an array of microorganisms. Several HDPs are known in mammals and humans, though the main ones are cathelicidins and defensins. Recent studies have shown HDPs' impact on several biological processes, in which they affect e.g. angiogenesis and wound healing. In this study four peptides were of interest, insulin, neurotensin, nisin and substance P. These peptides were investigated for their role in wound healing. Cells from a keratinocyte cell line (HaCaT) were subcultivated and a scratch assay was performed in a 48-well plate. The well plate was incubated with neurotensin, nisin and a control, and the scratch was measured at time (hours) 0, 4, 7 and 18. This was done in order to see the cell migration and thereby find out whether the peptides had an effect on wound healing. The average healing percentage was 65.4% for neurotensin, 75.1% for nisin and 65.0% for the control. Furthermore, RNA isolation, cDNA synthesis, PCR and gel electrophoresis were performed. However, as real-time polymerase chain reaction (qPCR) was not performed, the stimulatory effects of the selected peptides and their link to wound healing could not be concluded.

Key terms

Host defense peptides, antimicrobial resistance, wound healing, cytokines, immune system, epidermis, keratinocytes, insulin, neurotensin, nisin and substance P.

Abbreviations

AR: Antimicrobial resistance

cDNA: complementary Deoxyribonucleic acid

ddH_2O: double-distilled water

DNA: Deoxyribonucleic acid

DMEM: Dulbecco's Modified Eagle's Media

dNTPs: Deoxynucleotides (adenine, thymine, cytosine, guanine)

ECM: Extracellular matrix

EGF: Epidermal growth factor

ERK: Extracellular-signal-regulated kinases

EtBr: Ethidium bromide

FSB: Fetal Bovine Serum

GPCR: G-protein-coupled receptor

Grb2: Growth factor receptor-bound protein-2

hBD: human beta defensin

hCAP18: Human cationic antimicrobial peptide-18

HD: human defensin

HDP: Host defense peptides

HNP: Human neutrophil peptide

Ig: Immunoglobulin

IGF: Insulin-like growth factor

IL: Interleukin

IR: Insulin receptor

kDa: kiloDaltons

LAF: Laminar flow bench

LPS: Lipopolysaccharide

MEK: Mitogen-activated protein kinase kinase

mRNA: Messenger Ribonucleic acid

NT: Neurotensin

NTR: Neurotensin receptor

PCR: polymerase chain reaction

PDGF: Platelet-derived growth factor

PBS: Phosphate-buffered saline

qPCR: real time PCR

Raf: Rapidly accelerated fibro sarcoma

RNA: Ribonucleic acid

RPLP0: Ribosomal protein lateral stalk subunit P0

RTD: Rhesus Theta Defensin

SHC: Src homology 2 domain-containing

SOS: Son of sevenless

SP: Substance P

TBE: Tris/Borate/EDTA

TGF-ß: Transforming growth factor beta

TNF: Tumor necrosis factor

VEGF: vascular endothelial cell growth factor

Table of contents

1.0 Introduction

Humanity has been exposed to various fatal diseases throughout the centuries. In the early 20th century, antibiotics were discovered, which led to a decrease in mortality rate. However, various pathogens have developed resistant strains against antibiotics, (Aminov., 2010)(Davies & Davies., 2010) and the need for novel antimicrobial treatments have therefore become essential (Nijnik et al., 2010). Naturally occurring host defense peptides (HDPs) have attracted the attention in the medical industry due to their broad immunomodulatory properties and antimicrobial activity (Mansour et al., 2014). The immunomodulatory activities of HDPs include, among others, anti-inflammatory responses through altering signaling pathways, cell migration, angiogenesis, lipopolysaccharide (LPS) neutralization, cytokine regulation and collagen synthesis (Nijnik et al., 2010)(Mansour et al., 2014)(Diegelmann & Evans., 2004).

The immune system consists of multiple components including organs, tissues, cells and proteins, which protects the body through innate immune mechanisms that are capable of recognizing foreign microorganisms (Willey et al., 2014). Several cells such as keratinocytes, fibroblasts, macrophages, platelets, leukocytes and cytokines play a key role in the innate immune response and function to eliminate infections. Keratinocytes are the predominant cell type in the skin. They act as a protective barrier against the external environment and warn the body to danger by e.g. producing cytokines (Gröne, 2002). Cytokines are chemical messengers that influence the intensity and duration of immune responses (Prescott et al., 1999). Interleukins, a family of cytokines, are communicators between leukocytes and have been shown to stimulate the immune system responses (Tortora et al., 2010). Quantification of cytokine gene expression and protein levels is important when researching inflammatory -and immune responses. Changes in secreted protein levels of cytokines are correlated with changes in corresponding mRNA levels. This deciphers the mechanisms regulating the cytokine production keratinocyte culture (Qiagen, 2015)

The process of wound healing repairs and restores damaged tissue (Guo & Dipietro, 2010). It has three main phases: inflammation, proliferation and remodeling (Chen et al., 2012)(Liu et al., 2009a). The wound healing process is essential, as failure of the mechanisms, underlying the recovery of damaged tissues can lead to the formation of non-healing chronic wounds. Moreover, opportunistic pathogens, such as *Staphylococcus aureus*, are able to colonize the unhealed wounds leading to biofilm formation and toxin production. This results in damaged inflammatory responses (Mangoni et al., 2016). Therefore, investigating HDPs and their role in wound healing is the topic

of our bachelor project. In this project, the immune modulating role of 4 peptides: insulin, neurotensin, nisin and substance P will be investigated. Insulin is widely described in scientific literature due to e.g. its role in diabetic treatments, though less literature on the other peptides of our interest are published. Shortage of data on the four peptides and their role in wound healing suggest that it is a relatively new area and, considering the studies published so far, may play a big role in upcoming treatments.

1.1 Aim of the project

The aim of this project is to establish the immune modulating role of 4 peptides: insulin, neurotensin, nisin and substance P, in regard to cytokine regulation in a wound healing model and compare the findings with literature research.

1.2 Research questions

Do insulin, neurotensin, nisin and substance P have an effect on wound closure? What is the immune modulating role of those peptides on cytokine regulation in keratinocytes?

1.3 Reading guide

This project is divided into sections. Each section starts with an introduction shortly explaining the content. A glossary section can be found at the end of the project, in which scientific terms are explained. Moreover, the appendix contains protocols for the experiments as well as additional results, not shown in the results section. Due to time constrictions, only neurotensin and nisin were experimentally investigated.

2.0 Theory

2.1 Discovery and development of antimicrobial drugs

This section focuses on antimicrobial drugs, their discovery and function as well as the development and function of antimicrobial resistance.

Antibiotics are small chemical molecules, which have the capacity to interfere with the crucial pathways within pathogens and their life process by either annihilating, or inhibiting their growth (Drilica & Perlin, 2011). The "discovery void" started after the 1980's, when the mainstream approach on enlarging the discovery rate of antibiotics, had been through modifications of the existing antibiotics (Aminov, 2010). Despite the success of antibiotics against pathogens, evolutionary processes interfered, which resulted in microorganisms developing resistance to specific antimicrobial drugs (WHO, 2014). New antibiotics were discovered during the period between 1946 and the 1980's. This is known as the golden age (Aminov, 2010). Ever since antibiotics came on the market, the main use has specifically been on human and veterinary health. The benefits of antibiotics play a major role in the food industry not only to counteract foodborne pathogens and prevent them from spreading, but also to improve livestock. Antibiotics are usually used as the growth enhancers of healthy livestock (Sarmah et al., 2006).

2.1.1 Mechanisms of action

Antibiotics are effective against gram-positive and gram-negative pathogens, and function in various ways, as presented in Figure 1 (Lewis, 2013). The exploited targets that most antibiotics target are ribosomes, which are composed of the 50S and 30S subunits, cell wall synthesis, and DNA gyrase (Lewis, 2013). Perceiving it from a molecular perspective, the antibiotic Tetracycline for example, is profitable against both gram-positive and gram-negative pathogens and is extensively used in prophylaxis treatments in livestock and human health (Chopra & Roberts., 2001). This antibiotic has the capacity to inhibit protein synthesis of the pathogen by blocking the aminoacyl-tRNA to adhere on the 30S subunit of the ribosomal acceptor (A) site (Chopra & Roberts., 2001). In contrast, the antibiotic, Macrolides, binds to the 50S subunit of the ribosome and interferes with the ribosomal translocation by inhibiting the extension of nascent polypeptide chains (Shaikh et al.,2014). As previously mentioned, cell wall synthesis is thought to be one of the exploited targets for antibiotics, and penicillin is one of the listed antibiotics, that perform inhibition of wall synthesis (Yocum et al., 1980).

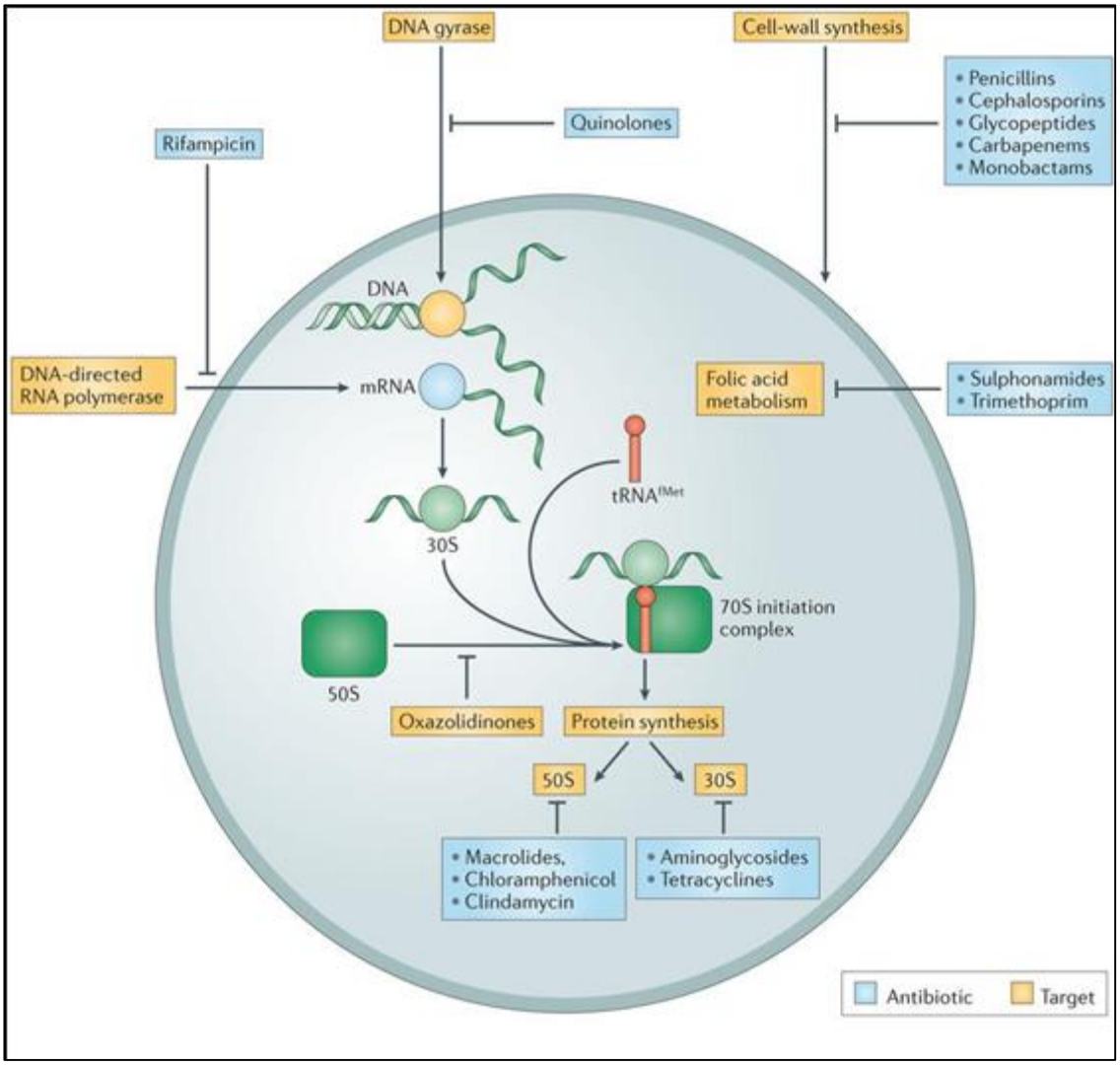


Figure 1 ***Antibiotics' modes of action****: The main exploited targets are the ribosomes which are composed of a 50S and 30S subunits , cell wall synthesis, and DNA gyrase or topoisomerase* (Lewis, 2013)*. As illustrated, Tetracycline and Aminoglycosides are the antibiotics that target the 30S subunit of the ribosome in bacteria, in contrast to Macrolides, Chloramphenicol and Clindamycin, which target the 50S subunit of the ribosome in bacteria. As previously mentioned, cell wall synthesis is thought to be one of the exploited targets of antibiotics, and penicillin as well as other antibiotics illustrated, performs this* (Yocum et al., 1980)*. The Folic acid metabolism functions by producing DNA and RNA in bacteria, and is targeted by Sulphonamides and Trimethoprim The DNA gyrase in bacteria is an enzyme which functions by catalyzing the ATP-dependent negative supercoiling of the DNA and is targeted by Quinolones* (Reece & Maxwell, 1991)*. Several other immense targets remain unexploited*(Lewis, 2013).

2.2 Emergence of antimicrobial resistance

Ever since penicillin came on the market, and before its extensive use, Alexander Fleming, in his 1945 Nobel Prize speech, warned that the bacterial pathogens could produce resistant strains against antibiotics (WHO, 2014).The prophylactic overuse of antibiotics has resulted in antibiotic resistance towards the healthcare of veterinary and human beings (Mathew et al., 2007). Hence, each time a new antibiotic was developed, resistance followed shortly after, resulting in less effective drugs.

Resistance has always been a part of the evolutionary process for pathogens, although according to studies, it has emerged by the selective pressure of the extensive use of antibiotics (WHO, 2014).

2.2.1 Mechanisms of resistance

Antimicrobial resistance (AR) and their mode of action are known to occur naturally within the population of pathogens. AR can arise by either a resistant gene, which is plasmid mediated, or by a random mutation on the bacterial chromosome (Dever & Dermody., 1991).The resistance mechanisms develop once the antibiotic enzymes are inactivated or degraded. There are three general mechanisms, in which antibiotic resistance can occur: (1) Antibiotic inactivation, by preventing the active molecule from recognizing its target, (2) Target modification or direct destruction of the compound, (3) Efflux pumps and permeability changes (Wright, 2005)(Dzidic et al.,2008). Antibiotics are known to have hydrolytically susceptible chemical bonds such as esters and amides. Certain enzymes have the ability to damage the antibiotic activity by cleaving and targeting these bonds (Shaikh et al., 2015).These enzymes are usually excreted by bacterial pathogens, resulting in inactivating, or hydrolyzing the antibiotics before they can reach their specific targets. β-lactamases are known hydrolytic amidases that cleave the β-lactam ring of both penicillin and cephalosporin and is known to be the most important mechanism of resistance (Dzidic et al., 2008)(Dever & Dermody., 1991). The second more important resistant mechanism is the target site of the modified antibiotic. This way, the antibiotic is unable to bind properly (Shaikh et al., 2015).

The efflux pumps are considered an essential resistant mechanism, which affect all classes of antibiotics, particularly the ones that inhibits protein and DNA biosynthesis (tetracycline, macrolides, and fluoroquinolones) (Dzidic et al., 2008). As illustrated in Figure 2 (Allen et al., 2010), efflux pumps are membrane proteins that function by exporting the antibiotics out of the cell. This contributes to a decreasing level of intercellular concentration of antibiotics (Nikaido & Pagès., 2012). If the outer membrane permeability is reduced, this will result in a depleted antibiotic uptake. Many efflux pumps are multidrug transporters and are known to be the latter contributing to multidrug-resistant bacterial pathogen (Dzidic et al., 2008).

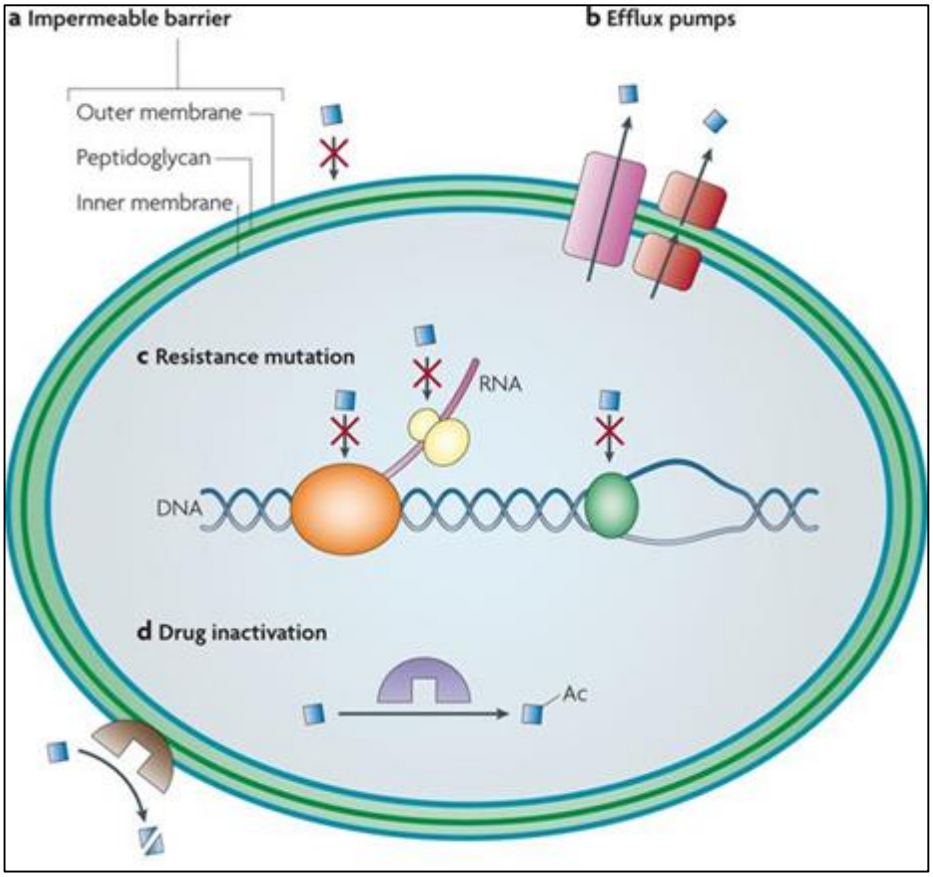


Figure 2 ***Impermeable barriers of bacterial cells*** (a) envelopes as the cause of resistance to certain antibiotics, due to the fact, that they either have an impermeable membrane, or lack the antibiotic target site. (Illustrated as the blue squares) The efflux pumps (b) are capable of secreting the antibiotics from the cell. Some can export antibiotics directly outside the cell (pink pump), whereas other pumps release them into the periplasm (red pump). Resistance mutations (c) are capable of modifying the protein target site of the antibiotic. Inactivation or modifications of antibiotics are either catalyzed by acetyltransferases (illustrated as purple) on aminoglycoside antibiotics, or by beta-lactamases (illustrated as brown) on beta-lactam antibiotics (d) (Allen et al., 2010).

2.3 Anatomy and physiology of human skin

The following section aims to provide the reader with an understanding of the anatomy and physiology of the human skin, also termed the integumentary system. The role and function of the three layers: the epidermis, dermis and the subcutaneous tissue of the skin are briefly described.

The human skin is a complex multi-layered tissue and the largest organ of the body with various functions. Most importantly, it forms a physical barrier to protect the body against microorganisms, ultraviolet radiation and toxic agents (McLafferty et al., 2012). The epidermis is the external layer of the skin and is a stratified epithelium that contains a number of cell populations e.g. keratinocytes, melanocytes, Langerhans and Merkel cells. These are illustrated in Figure 3 (Tortora & Bryan., 2009). 90% of the epidermis layer is composed of keratinocytes, which produce keratin, a long threadlike protein that protects the skin from heat, chemicals and microorganisms (McLafferty et al., 2012). Melanocytes are epithelial cells, which produce the pigment melanin, whereas Langerhans are dendritic cells involved in the activation of the immune system. Merkel cells are correlated with sensory neurons, detecting the sensation of touch (Tortora & Bryan., 2009).

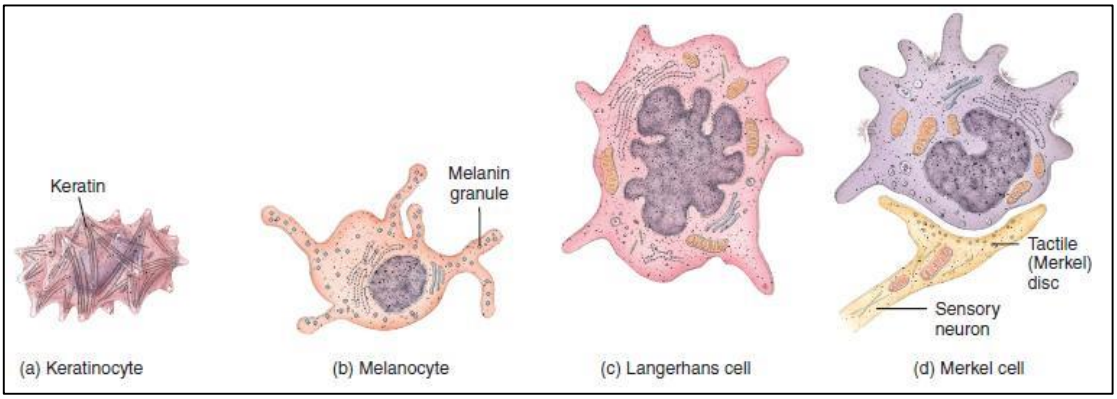


Figure 3 ***Cell types in the epidermis****. The epidermis layer contains keratinocytes, which functions by producing keratin as protective agent; Melanocytes, which contributes in producing the pigment melanin to the skin; Langerhans cells are dendritic cells, which functions by activating the immune system; and Merkel cells which functions by detecting the sensation of touch (Tortora & Bryan., 2009)*

The epidermis is comprised of four layers or strata, as illustrated in Figure 4 (Tortora & Bryan, 2009). (1) Stratum basale (2) Stratum spinosum, (3) Stratum granulosum, and (4) Stratum corneum. In addition, Stratum lucidum is found between the stratum granulosum and stratum corneum. It is only found in areas where the skin is thick, as e.g. the palms of the hands and soles of the feet (Rittié, 2016). Together, these layers are essential for the stages of cell maturation and their movement throughout the layers.

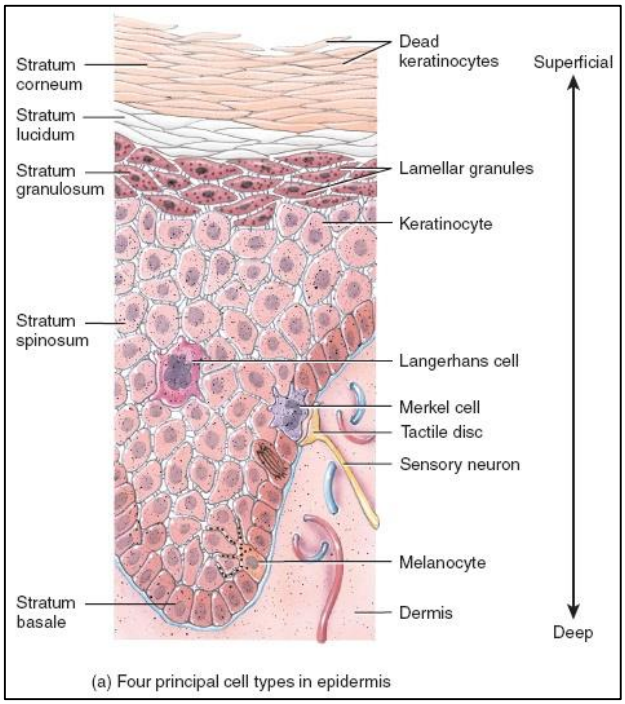


Figure 4 ***The layers of the epidermis.*** *The four layers: (1) Stratum basale (2) Stratum spinosum, (3) Stratum granulosum, and (4) Stratum corneum represent the different stages of cell maturation and their migration throughout the layers* (Tortora & Bryan, 2009).

2.3.1 Stratum basale

Stratum basale is the deepest layer of the epidermis. It contains melanocytes, Merkel cells and other cell types such as proliferative keratinocytes and stem cells. The stem cells undergo mitosis, and accumulate more differentiated keratinocytes. One of the newly formed keratinocyte cells will remain in the stratum basale, while the others slowly migrate to the surface in order to form the superficial layers of the epidermis (Tortora & Bryan, 2009)(Levy et al., 2007). This process is called keratinization and takes approximately four weeks in an average epidermis of 0.1mm thickness (Tortora & Bryan, 2009).

2.3.2 Stratum spinosum

The spinous layer, stratum spinosum contains Langerhans cells and melanocytes. This layer is superficial to the stratum basale. It is made of various layers of keratinocytes that have lost their proliferation potential and their ability to divide. They therefore become rounder and more thorn-like (Rittié, 2016). At a microscopic level, the cells of the stratum spinosum shrink and pull apart giving a thorn-like projection, where a bundle of tonofilaments is embedded into desmosomes that tightly draw the cells together again. This type of adjustment contributes with both strength and flexibility to the skin (Tortora & Bryan, 2009).

2.3.3 Stratum grunalosum

The granular layer, stratum granulosum is approximately the middle of the epidermis, and is composed of three to five layers of flattened keratinocytes. The flattened keratinocytes undergo apoptosis, making the keratinocytes unable to perform metabolic reactions. The cells in this layer become flattened and longer. The cells lose their nucleus and become more keratinized due to the presence of the protein, keratohyalin (Tortora & Bryan, 2009)(McLafferty et al., 2012).

2.3.4 Stratum corneum

The stratum corneum is known as the horny layer, and is the final outcome of keratinocyte maturation. It consists of 15-30 layers of flattened cornified cells known as corneocytes, which are replaced continuously by other cells from the deeper strata, and mostly contain the protein keratin (Rittié, 2016). The intercellular lipids from lamellar granules of the stratum granulosum, contributes to this layer by providing a natural and water-retaining barrier of the skin, hence preventing the cells from drying out (Tortora & Bryan, 2009)(McLafferty et al., 2012).

2.3.5 Dermis

The dermis is the second layer of the skin, located below the epidermis and above the subcutaneous tissue. Here, a base membrane zone is located, which functions by forming an interface between the epidermis and dermis, in order to provide nutrients and physical support for both tissues (Kolarsick et al, 2011). Furthermore, the dermis contains fibroblasts, mast cells, macrophages, and proteoglycans. The dermis is composed of two layers: a thin papillary layer and a thick reticular layer. The papillary layer contains areolar connective tissue enclosed by thin collagen and elastin fibers. The surface area of the dermis consists of dermal papillae, which are fingerlike projections that extend towards the surface of the epidermis. Blood vessels and nerve endings are embedded in this layer. The reticular layer is the second layer, which connects to the subcutaneous tissue, composed of thicker bundles of collagen, fibroblasts and elastic fibers. Blood vessels, hair follicles, nerves, nails sebaceous glands and sweat glands are embedded within this thick reticular layer of the dermis. Nevertheless, the reticular layer provides the skin with great extensibility and elasticity (Tortora & Bryan, 2009). The dermis helps comprising the bulk of the skin and because of its network of fibers it contributes to stretch and recoil, and has great tensile strength.

2.3.6 Subcutaneous tissue

The subcutaneous tissue, also known as the hypodermis is located beneath the epidermis, and is composed of loose connective tissue, fibroblasts, macrophages and adipocytes. The adipocytes are

grouped together in lobules, and separated by the connective tissue. The subcutaneous tissue functions as a storehouse of energy (Kolarsick et al., 2011).

The three layers of the skin comprise an efficient barrier to the external surface, allow transmission of sensory instruction, and distribute a significant role in maintaining homeostasis. Both epidermis and dermis collaborate by repairing and remodeling the skin, hence healing the wounds (Kolarsick et al., 2011).

2.4 Immune system

In this section, the resistance mechanisms of the immune system and the innate and adaptive immune responses are described. Furthermore, antibodies and lymphocytes are presented.

The immune system is comprised of different components and its main job is to defend and protect the body against foreign substances and invaders as e.g. microorganisms and infections. The main components of the immune system are proteins, organs, cells and tissues, which together comprise an environment that neutralizes invaders and thereby protects the body (Willey et al., 2014). The immune system's response to foreign invaders, has two main response mechanisms. These are known as the innate response mechanisms and the adaptive immune response (Willey et al., 2014). The innate response mechanisms are the first part of defenses and the main function is to produce antimicrobial chemicals as protection. The adaptive immune response is a resistance mechanism that also functions as defense against foreign substances. Though, it is more complex as antigens must be recognized in order to function (Martini et al., 2015). It can identify foreign substances, which triggers the immune response. These are referred to as antigens. The presence of antigens makes cells replicate and secrete glycoproteins, also referred to as antibodies (Willey et al., 2014). The central characteristic of the adaptive immune response is the memory (Martini et al., 2015).

The main function of antibodies is to inactivate antigens by binding to them. Figure 5A shows antigens and antibodies (Martini et al., 2015). Antibodies belong to a family, referred to as immunoglobulins (Ig), illustrated in Figure 5B. These immunoglobulins have different roles against invaders and are referred to as; IgG, IgM, IgA, IgE and IgD.

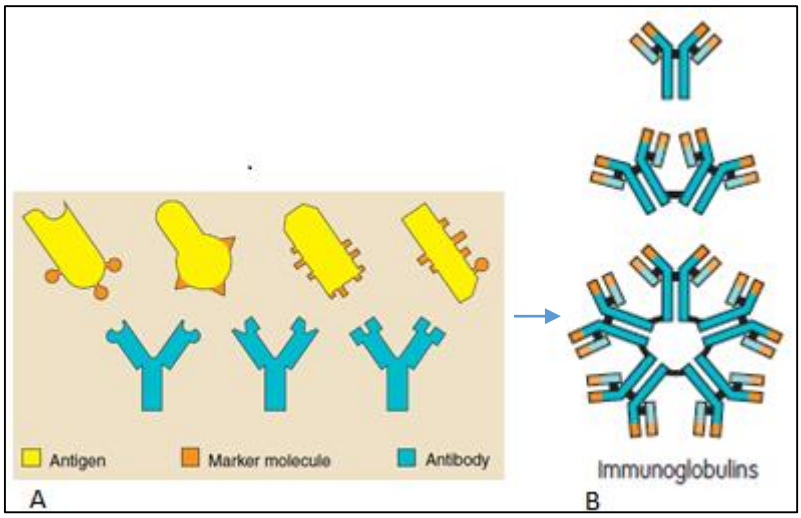


Figure 5 ***Structure of the antibodies and antigens****. Figure3A shows the structure of antigens, marked with yellow, marker molecules marked with orange and antibodies marked with blue. The antigens carry marker molecules that can recognize them and identify them as invaders. Figure 5B shows the structure of immunoglobulin, composed of several antibodies (Modified from* "National Cancer Institute," n.d.)

The immune system is located throughout the body, and is made up of lymphoid organs, in which the lymphocytes are the essential cells. Lymphocytes are developed in bone marrow; this is illustrated on Figure 6 (Willey et al., 2014). They function in both the innate response mechanisms and in the adaptive immune response.

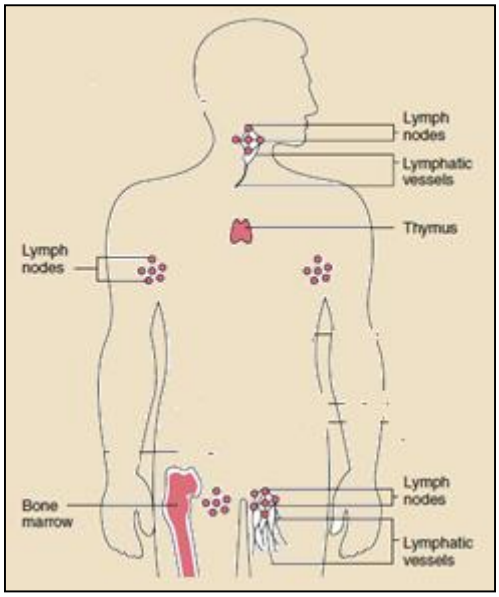


Figure 6 ***Organs of the immune system*** *shows some of the main organs of the immune system; the lymph nodes, the bone marrow, the thymus, and the lymphatic vessels. These are heavy marked with pink (Modified from* "National Cancer Institute, n.d.)

The lymphocytes are divided into two main cell populations referred to as; T- and B-cells. Both T- and B-cells differentiate from their respective lymphoid cells and exit the bone marrow through the blood vessels (Martini et al., 2015). T-cells mature in the thymus gland, also shown in Figure 7 and bind to a T-cell receptor where it is activated. Once activated, the cell can further differentiate into

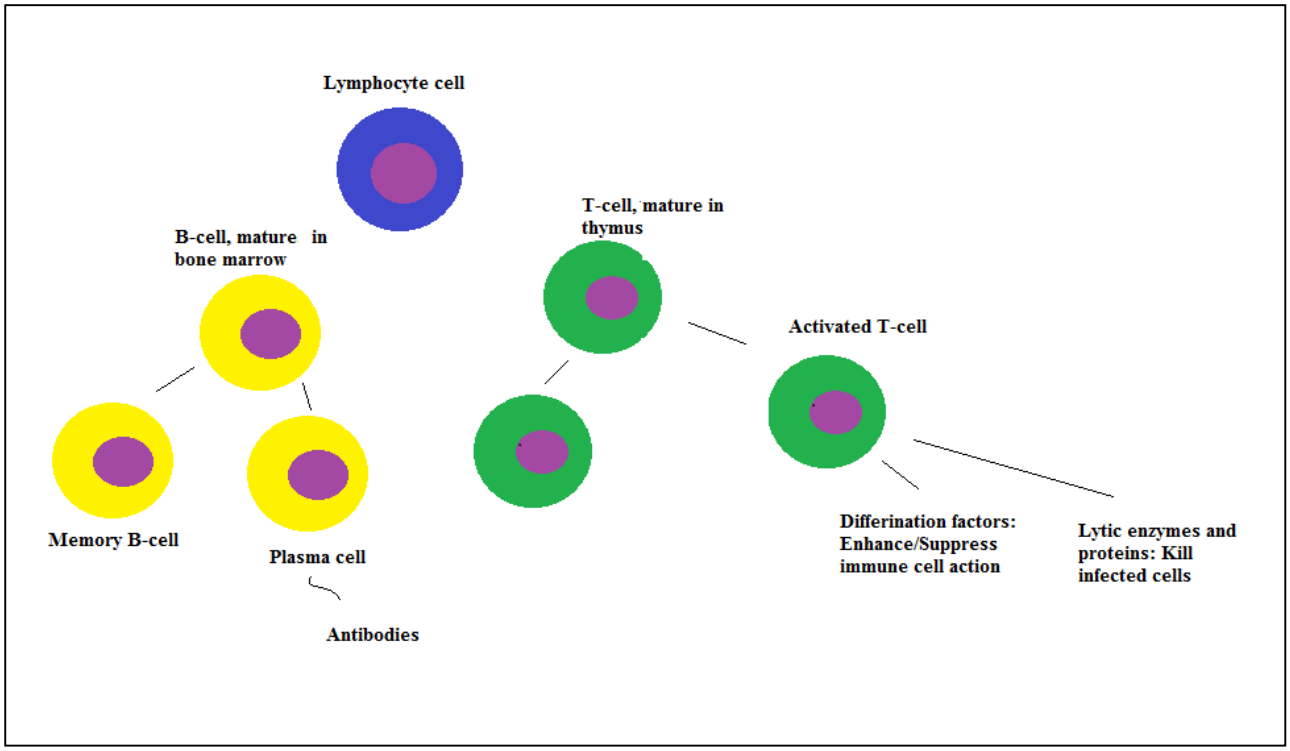


Figure 7 ***cells of the immune system*** *shows an illustration of B-cells marked with yellow, and T-cells marked with green, arisen from the same cell. The lymphocyte cell, marked with blue. B-cells mature in bone marrow, where they become plasma cells, that can produce antibodies or memory cells that can recognize foreign substances. T-cells mature in thymus, and develop to memory cells. The mature T-cells has differentiation factors and lytic enzymes/proteins that defends against foreign (Modified, inspired by* (Willey et al., 2014).

memory cells that can respond to foreign invaders. This is illustrated Figure 7. T-helper cells (T_H) are a subpopulation of T-cells that respond to antigens by producing cytokines and lymphokines. The latter can activate various phagocytic cells, enabling them to kill microorganisms more efficiently (Kuby, 1992). B-cells mature in bone marrow and diffuse to lymphoid organs, where they are activated. When B-cells are activated, they are called plasma cells and their main function is to secrete antibodies. Furthermore, the activated B-cells can produce memory cells that can act upon antigens, as illustrated in Figure 7 (Willey et al., 2014).

Another essential part of the immune system is the cytokines. They will be further described below.

2.5 Cytokines

This section focuses on the various cytokine families, their functions and certain interleukins.

Cytokines are soluble glycoproteins that regulate the intensity and duration of immune responses. One of the roles of these small proteins (~5-10kDa) is to recruit macrophages, dendritic cells and other defensive cells to isolate and eliminate the microbes as part of the inflammatory response. Moreover, cytokines mediate the communication between various immune system cells in order to create a response to a stimulus. However, a cytokine can only act upon cells that have a receptor for that certain cytokine (Tortora et al., 2010). There are few families of cytokines, which are grouped according to their function or according to the immune cell they are produced by (Prescott et al., 1999).

Interleukins (ILs) are communicators between leukocytes. The role of interleukins in stimulating the immune system has suggested their use as therapeutic agents. The cell source and functions of interleukins IL-6, IL-8 and IL-10 will briefly be described below. Activated T_H cells (2. subset), macrophages, monocytes, fibroblasts and endothelial cells secrete IL-6. Its function is to increase secretion of antibodies by plasma cells. Furthermore, together with IL-1, IL-6 co-stimulates T_H-cell activation. Additionally, it induces the growth of T-cells, B-cells, keratinocytes and nerve cells. IL-8 is secreted by primary monocytes and macrophages and has various effects on neutrophils. It chemo-attracts neutrophils, induces their adherence to vascular endothelial cells and aids their migration into tissue spaces toward an IL-8 concentration gradient. Moreover, IL-8 is a chemoattractant for granulocytes and inhibits their adhesion to endothelium. IL-10 is an anti-inflammatory cytokine secreted by T_H cells (2. subset). It suppresses cytokine production by 1. subset of T_H. This interleukin plays a central role in regulating humoral and cell-mediated responses. IL-10 also enhances B-cell survival, proliferation, and antibody production (Prescott et al., 1999)(Kuby, 1992).

Chemokines another family of small cytokines, stimulate cell migration and attract phagocytic cells and lymphocytes. Chemokines play a central role in the inflammatory response (Prescott et al., 1999). Moreover, there are cytokines, which main function is to protect cells from viral infection. They are called interferons and a number of them are commercialized as treatment to conditions such as hepatitis and some types of cancer (Tortora et al., 2010). The tumor necrosis factor (TNF), another family, are well-studied cytokines of natural immunity. A major TNF-α, mediates the host response to LPS, present in gram-negative bacteria. When TNF-α is released by mononuclear phag-

ocytes due to stimulation by LPS, it stimulates an increase in the number of B-cells and secretion of IL-1, IL-6 and IL-8. Last but not least, the family of hematopoietins functions in controlling the pathways by which stem cells develop into white and red blood cells (Prescott et al., 1999)(Kuby, 1992).

Cytokines can also stimulate cells to produce more cytokines. While this normally is a positive function, it can lead to a harmful overproduction of cytokines, termed a cytokine storm. That may happen when the feedback loop of the concentration of those proteins gets out of control. A cytokine storm can result in significant tissue damage and appears to be linked to multiple diseases such as influenza, graft-versus-host disease and sepsis as a pathological factor (Tortora et al., 2010).

2.6 Host defense peptides

This section describes HDPs, their main functions, cathelicidins and defensins. Furthermore, LL-37 is presented.

Antimicrobial peptides also referred to as HDPs, are molecules found in all domains of life. As AR has become a major problem, antimicrobial agents have been an important component as "nature's antibiotics" (Hannock & Sahl, 2006). HDPs' primary function is to inhibit the microorganisms from overgrowing (Sidky et al., 2012). More than 100 HDPs are found in the human body. They are identified from tissues and various epithelial surfaces (Wang, 2008). Two major groups of HDPs are identified in humans and mammals. One group has helix structures and are referred to as cathelicidins. The other group has β-sheet structures and is named defensins (Wang, 2008). Both groups are expressed in mucosal epithelium (Mansour et al., 2014). They are known for their broad spectrum of antimicrobial activity because they play a role in helping various health problems as e.g. skin diseases, wounds, dermatitis and acne (Sidky et al., 2012).

Cathelicidin is located on chromosome 3 and is referred to as human cathelicidin antimicrobial peptide (*CAMP*). This gene encodes for a protein with the property of an inactive precursor protein named human cationic antimicrobial peptide-18 (hCAP18). hCAP18 has a C-terminal named LL-37 (Guaní-Guerra et al., 2010). Defensins are further categorized into three groups; α-defensins, β-defensins and θ-defensins. α-defensins are expressed in humans. They are named human neutrophil peptides (HNP) because of their expression in neutrophil granules (Guaní-Guerra et al., 2010)(Mansour et al., 2014). These are referred to as HNP [1-4]. Five and six are referred to as human defensins (HD5 and HD6), and are expressed in paneth cells. Additionally, there are six β-defensins expressed in epithelial cells, named human beta defensins, (hBD) [1-6]. The last group of defensins, θ-defensins, are found in non-human primates and are expressed in leukocytes. These are known as rhesus theta defensins (RTD) and three are known and referred to as RTD (1-3) (Guaní-Guerra et al., 2010). The above-mentioned is illustrated in Table 1.

Table 1 ***key features of the cathelicidins and defensins****. 3 categories are shown: AMPs , sources of the AMPs and the regulation and induction stimuli of them. Seven AMPs are shown: LL-37, HNP(1,2,3 and 4), HD 5 and 6 and HBD (1,2,3 and 4) (Modified from* (Guaní-Guerra et al., 2010).

Table 1 Key features of cathelicidin and defensins.

AMPs	Source	Regulation and induction stimuli
α-Helical		
LL-37	Epithelial cells: gut, urinary tract, respiratory tract, skin. Leucocytes: neutrophils, monocytes mast and myeloid cells.	Constitutive and inducible by: VD3 (keratinocytes, monocytes, neutrophils) LPS (synergic effect with VD3) Butyric acid (colonocytes)
Cystein rich		
α-defensins		
HNP-1,2,3,4	Neutrophil azurophilic granules	Constitutively expressed.
HD-5,6 (Cryptidins)	Paneth cells and epithelial cells of the female genitourinary tract.	Constitutively expressed. Bacterial products (muramyl dipeptide)
β-defensins		
HBD-1	Lung and urinary tract epithelial cells	Constitutively expressed.
HBD-2	Skin, lung, gut and genitourinary tract epithelial cells.	Inducible expression by: IL-1α, IL-1β, Gram (+), Gram(−) TNF, *C. albicans*, LPS, LAM.
HBD-3	Several epithelial surfaces. High concentration in saliva and vaginal swabs.	Inducible expression.
HBD-4	Testicles, uterus and stomach.	Inducible expression.

The main function of HDPs is the anti-inflammatory response by altering signaling pathways. Both cathelicidins and defensins act directly on monocytes and neutrophils. Cathelicidins are involved in releasing monocyte-chemoattractant proteins 1 and 3 (Mansour et al., 2014). These are necessary for the immune system, in which cells work by removing invading microorganisms (Guaní-Guerra, et al. 2010). Cathelicidin has also been shown to block the release of tumor necrosis factor α (TNF-α), which is a pro-inflammatory cytokine induced by Lipopolysaccharide (LPS) and Lipoteichoic acid in macrophages and monocytes (Mansour et al., 2014)(Guaní-Guerra et al., 2010).

HDPs have been investigated for their potential as therapeutic agents. The nature of HDPs seems to be active against both gram-positive and -negative bacteria, in viruses and fungi. Furthermore, according to Mansour and colleagues (2015) they are primarily involved in two main processes: bacterial membrane integrity and inhibition of essential processes including RNA, DNA, protein-synthesis, cell division and cytosolic enzyme inhibition (Mansour et al., 2014).

2.6.1 LL-37

LL-37 is known as the only cathelicidin-derived antimicrobial peptide identified in humans. The reason behind its name is due to the fact that it starts with two leucine residues, and contains 37 amino acids (Dürr et al., 2006)(Kościuczuk et al., 2012). LL-37 is the short form of FALL39 deter-

mined as the mature peptide, and hCAP18 is its precursor (Kościuczuk et al., 2012). LL-37 is found throughout the body, and is expressed in different cells and tissues such as epithelial skin cells, leukocytes such as T-cells, NK cells and B-cells, monocytes, and neutrophils, and also in the gastrointestinal tract as well as in the respiratory tract (Dürr et al., 2006) (Kościuczuk et al., 2012). It has been identified as a HDP, released in high levels by degranulated neutrophils during inflammation (Guaní-Guerra et al., 2010). The LL-37 peptide has been shown to have a broad spectrum of antimicrobial activity, and additional defense roles. Regarding its defensive roles, it has been shown that LL-37 can regulate the inflammatory response, and has a chemotactic activity that can chemoattract T-cell leukocytes, neutrophils and monocellular cells of the adaptive immune system to infection and wound sites (Dürr et al., 2006).

2.7 Wound healing

In this section, the three phases of wound healing: inflammation, proliferation and remodeling, are described. Additionally, the role of HDPs in wound healing are explained.

2.7.1. The three phases of wound healing

Wound healing is a process where the main function is the repair and restoration of the integrity and homeostasis of the tissue after injuries. However, factors as e.g. infections can hinder the wound healing process causing impaired tissue repair (Guo & Dipietro, 2010). Wound healing has three main, time overlapping phases: inflammation, proliferation and remodeling (Chen et al., 2012). Figure 8 shows an overview of the three phases in wound healing, the cells involved and their actions.

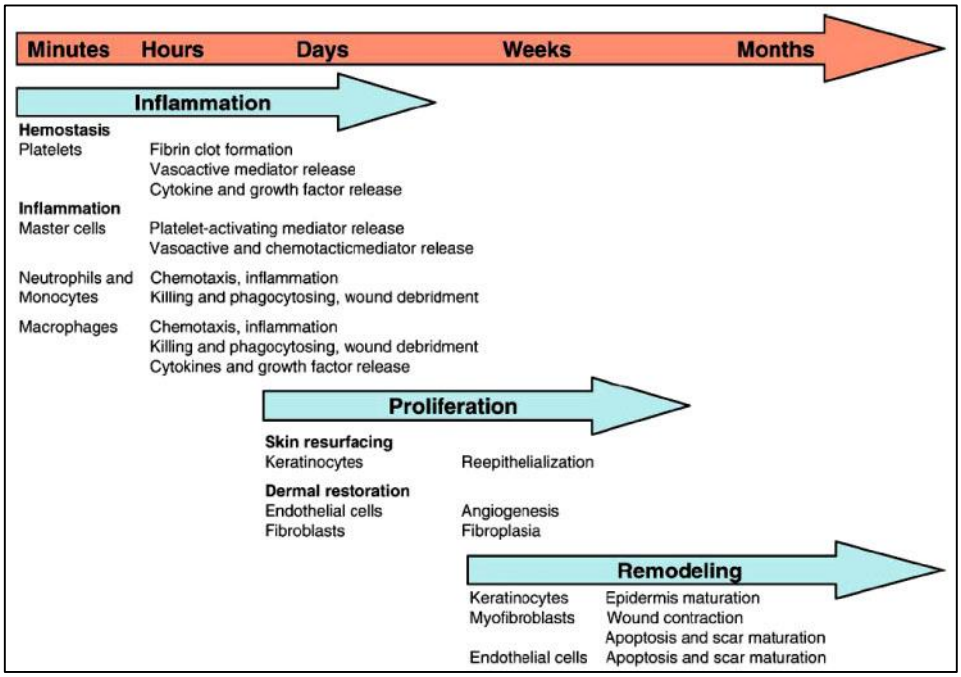


Figure 8 ***Overview of the three major phases in wound*** *healing illustrates an overview of the three phases: inflammation, proliferation and remodeling, in wound healing. Below each phase, the cells involved in the process along with their actions are shortly described. The inflammation phase includes platelets, master cells, neutrophils, monocytes and macrophages. The proliferation phase includes keratinocytes, endothelial cells and fibroblasts and the remodeling phase includes keratinocytes, myofibroblasts and endothelial cells.*[*http://e-dmj.org/search.php?where=aview&id=10.4093/kdj.2009.33.2.83&code=0004KDJ&vmode=PUBREADER*](http://e-dmj.org/search.php?where=aview&id=10.4093/kdj.2009.33.2.83&code=0004KDJ&vmode=PUBREADER)

The human body can withstand several varied injuries such as blunt trauma, penetrating trauma and burn trauma. These insults trigger an arranged sequence of events involved in the healing process, distinguished by specialized cells moving into the site of injury. First to arrive are the inflammatory cells and platelets, who signal for the need for a new blood supply and connective tissue cells. In the proliferative phase, connective tissue cells and fibroblasts migrate into the injury site, replacing the

blood clot with collagen. In the human body, collagen is highly abundant and accounts for 30% of the total protein. In healthy tissue, collagen provides structure, integrity and strength; therefore, in disrupted tissues following injury, collagen is needed to restore anatomic function and structure as well as to repair the defect. An insufficient collagen amount deposited into an injury site results in a weak wound that may gap. Too much collagen deposited into a site of injury and normal anatomical function is jeopardized, structure is lost and fibrosis can occur. In the final remodeling phase, the new collagen becomes organized and cross-linked and scar tissue matures (Diegelmann & Evans, 2004).

The healing process begins with hemostasis. Tissue damage causes extravasation of blood components as well as blood vessel disruption. As blood reaches the site of injury, platelets encounter extracellular matrix (ECM) components. This interaction triggers the release of clotting factors along with cytokines and growth factors such as the platelet-derived growth factor (PDGF) and TNF-β that attracts as well as activates fibroblasts and macrophages. The blood clot consists of platelets embedded in cross-linked fibrin fiber and serves as a transitory protection for the damaged tissues. Additionally, it reverses hemostasis. However, in the absence of hemorrhage, when blood escapes the circulatory system, platelets are inessential to wound healing (Diegelmann & Evans, 2004)(Martin, 2014)(Singer & Clark, 1999).

In response to the surface alterations in the lining capillaries of the endothelial cells, monocytes and neutrophils are drafted from the blood circulation to the injury site. This is the beginning of the inflammatory phase (Martin, 2014). Neutrophils infiltrate the site of injury, cleansing the region for bacteria and foreign particles and are then phagocytosed by macrophages. Simultaneously, monocytes infiltrate the injury site in response to particular chemo-attractants, such as monocyte chemoattractant protein 1 or ECM protein fragments. When infiltrating, monocytes become activated macrophages, releasing certain growth factors, e.g. PDGF, which initiates the development of granulation tissue. Using their integrin receptors, macrophages bind to particular ECM proteins, which stimulates phagocytosis. Adherence to the ECM, induces macrophages and monocytes to express PDGF, TNF-α and colony-stimulating factor 1, a necessary cytokine for macrophage and monocyte survival. As macrophage-depleted animals show faulty wound healing, macrophage -and monocyte-derived growth factors appear to play a crucial role in the initiation and reproduction of novel tissue development in wounds. Hence, they seem to be imperative for the inflammation-repair transition (Singer & Clark, 1999).

The presence of growth factors, cytokines and macrophages suggest the ending of the inflammatory phase and the initiation of the proliferative phase (Diegelmann & Evans, 2004). In the beginning of the proliferative phase, keratinocytes migrate and proliferate at the edges of the wound. Subsequently, dermal fibroblasts proliferate in the wound region where they deposit ECM into the provisional wound matrix. The provisional matrix contains fibrin and provides a frame to guide cells into the site of injury, stimulating differentiation, proliferation and the synthesis of new ECM. Due to the contractile phenotype of fibroblasts, they can convert into myofibroblasts, which are involved in wound contraction. This wound contraction process is part of the remodeling phase (Werner & Grose, 2003). Along with the progression of the proliferative phase, the transforming growth factor β (TGF-β) released by macrophages, platelets and T-cells are considered a master signal as it regulates multiple fibroblast functions. TGF-β has three effects on ECM deposition; it stimulates the protease inhibitor named tissue inhibitor of metalloproteinase, decreases protease secretion of the proteases accountable for matrix breakdown, and increases the total production of matrix proteins by increasing transcription of the genes coding for fibronectin, proteoglycans and collagen (Diegelmann & Evans, 2004).

Multiple other responses are activated during the healing process. Fibroblasts, vascular endothelial cells, epidermal cells and macrophages produce TGF-β, basic fibroblast growth factor and vascular endothelial cell growth factor (VEGF), which stimulates angiogenesis (Diegelmann & Evans, 2004). Angiogenesis generates the development of novel blood vessels and nerve sprouting appears at the edge of the wound (Werner & Grose, 2003). A growing need for nutrients and oxygen develops as a result of the high metabolic activity at injury site. Local factors such as reduced oxygen tension and low pH in the microenvironment of the site of injury, initiate the release of the necessary nutrients and oxygen to make the novel blood supply. After a skin rupture, re-epithelization is needed. Epithelization is stimulated by TNF-α and epidermal growth factor (EGF), both of which are produced when platelets, macrophages and keratinocytes are activated in wounds. As the epithelial process completes, the scab is removed by enzymes dissolving the base attachment. The novel connective tissue at the injury site is referred to as granulation tissue as the several capillaries have a granular appearance. This is the beginning of the final phase, the remodeling phase, in which the granulation tissue transitions into a mature scar. This process is characterized by its continual collagen catabolism and synthesis. Currently, multiple collagen types have been identified though in skin scar tissue, type I is predominant. The enzyme, lysyl oxidase, is found in extracellular spaces. Its purpose is to form stable collagen-fibril cross-links by acting on collagen. The intra-intermolecular

cross-links increases as collagen matures and is significant for the strength and stability of collagen over time. The developed scar tissue lacks subordinate parts such as sweat glands and hair follicles and is therefore mechanically insufficient (Diegelmann & Evans, 2004).

The majority of skin wounds heal efficiently and quickly within 7 to 14 days, though it is depending on the size. However, the final result is not functionally nor visually perfect. At the injury site, lost epidermal subordinate parts do not regenerate and after the healing process ends, if the collagen matrix has been insufficiently reconstructed, a connective tissue scar remains (Martin, 2014). In healthy tissue, dermal collagen is a strong molecule with a tensile strength, on a per weight basis, close to steel. Contrarily, in scar tissue, the molecule is smaller with a tensile strength that does not reach normal, and a healed wound is therefore weaker than the surrounding healthy tissue (Diegelmann & Evans, 2004).

2.7.2 The role of growth factors and HDPs in wound healing

The roles of endogenous growth factors in the healing process is still only partially known. Their functions have only been somewhat explained based on functional cell culture data or descriptive expression studies. Moreover, several growth factors' *in vivo* functions remain unverified (Werner & Grose, 2003). Furthermore, a key aim in wound healing is to clarify how skin can be influenced to rebuild damaged tissue to perfection (Martin, 2014).

Studies have shown that HDPs play an essential role in wound healing, where both cathelicidins and defensins are involved (Guaní-Guerra et al., 2010). When a skin injury occurs, the expression of HDPs is increased. This increased expression leads to up-regulated synthesis by keratinocytes. In epidermal keratinocytes, an inducible and constant expression of hBD-2 and -3, and hCAP18 have been observed. At an injury site, hCAP18/LL-37 is stimulated by insulin-like growth Factor (IGF) 1 and TNF-α. In other epithelia, hCAP18 is upregulated in affiliation with injury and inflammation, which supports the idea of the suggested role in barrier defense. hCAP18 plays a significant role in wound healing as it activates fibroblasts and epidermal cells and thereby forming chemo-attract macrophages and fibroblasts and granulation tissue (Heilborn et al., 2003)(Steinstraesser et al., 2008)(Guani-Guerra et al., 2010). A study performed by Heilborn and colleagues (2003) showed that during re-epithelialization of human organ cultured skin, hCAP18 is induced. Additionally, the study showed antibodies against LL-37 prevented re-epithelialization, though it was concentration dependent. This suggests a vital role in wound healing (Heilborn et al., 2003). Additionally, LL-37 plays an important role by e.g. mediating angiogenesis and the hBD-2 defense stimulates prolifera-

tion and migration of the immune system's endothelial cells resulting in wound healing (Steinstraesser et al., 2008)(Heilborn et al., 2003)(Guaní-Guerra et al., 2010). Figure 9 shows an overview of HDPs role in different processes in regard to wound healing.

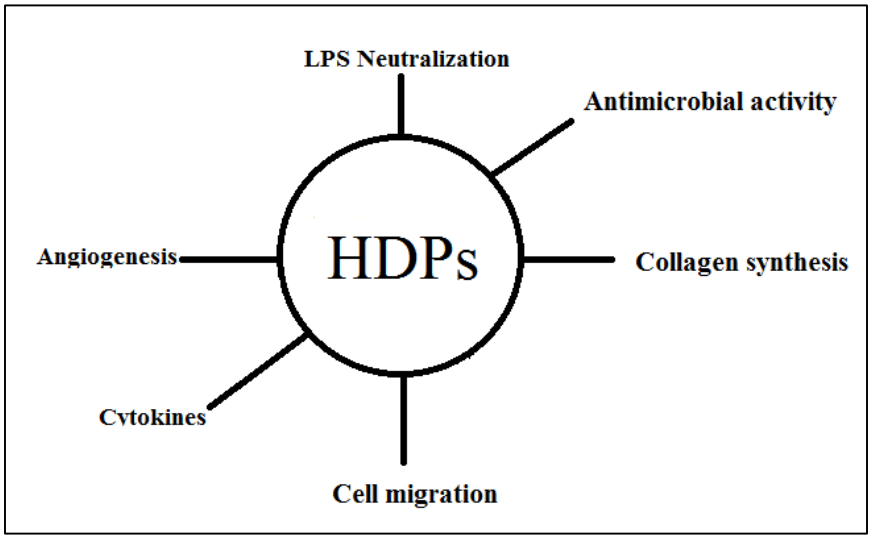


Figure 9 ***An overview of HDPs' multiple effects*** *Shows a self-made overview of multiple processes HDPs affect. These include antimicrobial activity, collagen synthesis, cytokines, cell migration, angiogenesis and LPS neutralization.*

3.0 Peptides

In this section, the four peptides: insulin, neurotensin, nisin and substance P, chosen for the project are described. For each peptide, general introduction, structure presentation and its relation to wound healing are presented. Abundance of before mentioned information vary from peptide to peptide, as the use of peptides in relation to wound healing is a relatively new topic.

3.1 Neuropeptides

Neuropeptides are minor molecules produced throughout the human body by multiple cells. When neuropeptides are released under inflammatory circumstances they are among others, capable of altering the activity of cells (platelets, neutrophils, macrophages, lymphocytes, dermal fibroblasts), that promote healing. The amplitude of neuropeptide-mediated effects possibly differ in inflammatory disorders and in healthy tissue. This is due to the fact, that cell populations and receptor levels that express neuropeptide receptors can be different. Furthermore, interactions with other mediators participating in tissue restitution such as cytokines and growth factors may modify neuropeptide-mediated effects (Brun, et al. 2004).

3.2 Insulin

The peptide hormone insulin is secreted by β-cells in the pancreas, when plasma glucose levels rise. It is a key metabolism-regulating hormone, which allows the human body to use glucose for energy or to store it in the liver for future use. Insulin's primary function is to maintain low blood glucose levels and counteract various hyperglycemia-generating hormones. Furthermore, insulin increases the transport of amino acids into cells, reduces lipolysis and stimulates lipogenesis. Insulin stimulates cell growth, protein translocation, cell replication and DNA synthesis. Furthermore, it modulates transcription, which it has in common with relaxin and (IGF) 1 and 2 (Hess-Fischl, 2016)(King, 2015).

3.2.1 Insulin structure and the insulin receptor

The primary structure of a human insulin molecule consist of two polypeptide chains (A and B) with 51 amino acid residues. Two disulfide bonds link the two chains. The A-chain has an additional disulfide loop that lies between A6 an A11 (Wang & Pearlman, 1993). The insulin receptor (IR) is a multi-subunit glycoprotein and is one of the most abundant cell surface receptors in the human body. The IR's are found various places for example on erythrocytes, myocytes, in brain tissue and on adipocytes. IR binding sites have among others, been found on both keratinocytes and dermal fibroblasts in unconnected populations with high and low affinity. Having low as well as high affin-

ity binding sites present allows the IR to vary its response to varying insulin concentrations in the blood (Hrynyk & Neufeld, 2014).

3.2.2 Receptor structure & signal transduction

The IR is a transmembrane receptor comprised of two α-subunits and two β-subunits connected by disulfide bonds. The IR receptor α-subunits are positioned on the extracellular part of the receptor (Lee & Pilch, 1994). The β-subunits holds an extracellular region, a transmembrane domain and a cytosolic region. Each αβ monomer has two binding sites: site 1 and site 2. When insulin binds to site 1 on one of the αβ monomers, site 2, on that same αβ monomer, becomes available for ligand binding which can activate the receptor. This is possible due to the high-affinity cross-linking reaction that occurs as insulin binds to site 1. This binding regime shows that the IR affinity for a second ligand decreases when the first ligand binds to the receptor. IR therefore exerts a negative cooperativity (Hrynyk & Neufeld, 2014). Once insulin is bound to the IR, the subunits form a single dimer and the signaling cascade begins. First, the intracellular tyrosines positioned on the receptors β-subunits are phosphorylated. The phosphorylated tyrosines then act as docking sites, allowing the Src-homology-2 domain containing (SHC) protein, in the cytosol, to bind through its Src-homology-2 domains and transduce the signal through various messenger molecules including growth factor receptor-bound protein-2 (Grb2). Next Grb2 uses a set of proteins, son of sevenless (SOS), to activate the Ras protein, which uses members of the GTP-binding protein family, Raf, MEK and ERK to carry the signal. Lastly, ERK translocates into the nucleus to tell the cell to proliferate and replicate DNA in order to repair damaged tissue and wound healing. Furthermore, IR activation stimulates two other pathways, glucose uptake and biomacromolecule synthesis, both of which necessary to support repair of damaged tissue (Hrynyk & Neufeld, 2014). Figure 10 shows the signaling cascade described above.

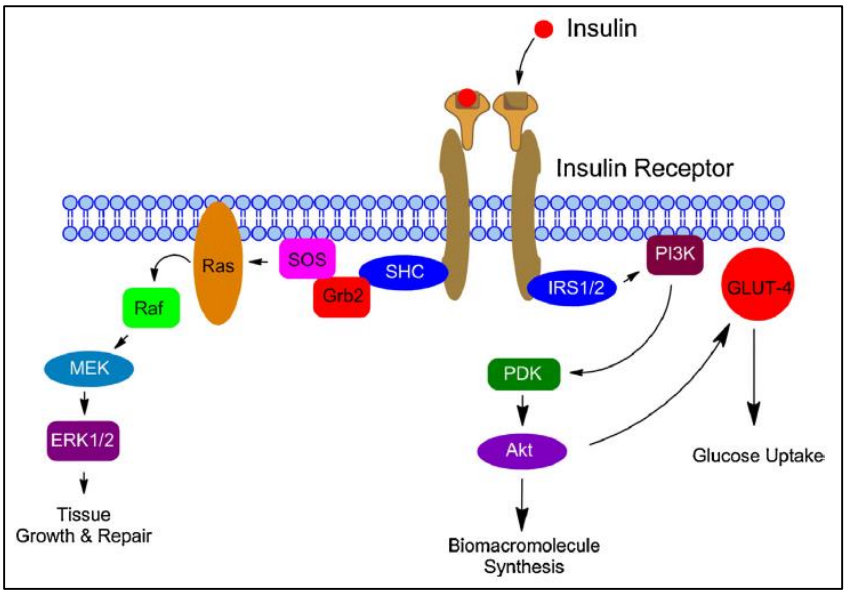


Figure 10 ***The insulin receptor and the signaling cascade*** *shows the insulin receptor with binding sites on the α units on the extracellular and the β units crossing into the intracellular. It shows the src homology 2 domain containing (SHC) protein, Grb2, SOS, Ras, Raf, MEK and ERK, all of which are part of the signal transduction pathway for tissue growth and repair. Furthermore, it shows the insulin receptor substrates 1/2 (IRS1/2), the PI3K, PDK, Akt and GLUT-4, all signaling messengers that are part of the signal transduction pathways used in glucose uptake and biomacromolecule synthesis. (Hrynyk & Neufeld 2014).*

3.2.3 Insulin in wound healing

In the early beginning of the 20th century, insulin was used for non-diabetic purposes (Liu et al., 2009b). Several animal models have shown that a continuous treatment of insulin expedites the healing of skin cuts and healing from fractures (Chen et al., 2012). Insulin has also been used to accelerate skin burn wounds as well as bone healing in rats (Liu et al., 2009a). A study performed by Chen and colleagues (2012) demonstrated that a small dose of topical insulin application advanced healing from incision wounds in rabbits(Chen et al., 2012). In the 1960's, medics began to use insulin when treating human diabetic wounds. More recently, the use of an insulin spray when treating patients with diabetic ulcers has been a success. Apart from *in vivo* studies, the results of insulin experiments with cultured cells have demonstrated that insulin expedites the growth rate of fibroblasts. This suggests that insulin may function as a growth hormone. Regardless of the compelling evidence implying that insulin stimulates wound healing and thus reduces wound closure time, the underlying mechanisms are yet to be understood (Liu et al., 2009b).

A study by Liu and colleagues (2009b) presented evidence that when insulin is topically applied to skin wounds, it stimulates the maturation of healing tissues and advances re-epithelialization. Figure 11 below shows skin excision wounds in mice, one control (saline) and one with insulin, applied every second day (Liu et al., 2009b).

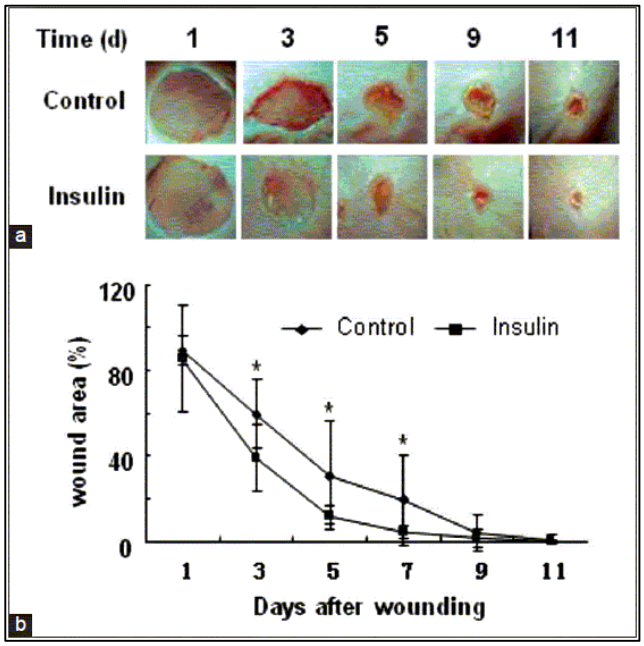


Figure 11 ***Insulin's effect on wound healing*** *Shows how Insulin expedites wound healing. C57BL/6J mice were used, excision wounds were performed and the healing process was monitored over days. (a) shows images of the wounds. On top is the control treated with a 30µl saline solution and below is the one treated with 0.03µg insulin per 30 µl saline solution every second day. (b) Every second day the wound area was quantified. It was then expressed in the graph as the percentage (%) of the original wound area. Insulin substantially reduced the wound area (Liu et al. 2009b)*

The literature used in this section indicates that insulin advances wound healing and suggest that it can be part of upcoming medicine used for wound healing. However, the literature also states that underlying mechanisms are yet to be understood.

3.3 Neurotensin

Neurotensin (NT) is a short, 13 amino acid, bioactive neuropeptide found in the nervous system, cardiovascular system, peripheral tissues and in the gastrointestinal tract. It regulates inflammatory processes in the gastric system and in the lungs (Moura et al., 2014a). NT functions both as a neurotransmitter in the nervous system and as a hormone in the periphery. As a hormone, NT is an endocrine, as well as paracrine modulator of the cardiovascular system and the gastrointestinal tract. Additionally, NT acts as a growth factor on several healthy, and cancer cells. As a neurotransmitter, NT regulates anterior pituitary hormone secretion, dopamine transmission and induces both analgesic and hypothermic effects in the brain (Vincent et al., 1999).

3.3.1 Neurotensin receptors

There are three NT receptors known to date. NT receptor 1 (NTR1) and NT receptor 2 (NTR2) have low and high NT affinity, respectively. They contain seven transmembrane helices and are both part of the class A G-protein coupled receptor (GPCR) family (Moura et al., 2014a). NT receptor 3 (NTR3) is part of the sortilin family and has a single transmembrane domain. NTR3 is an intracellular, non-GPCR. The majority of NT effects are facilitated through NTR1, which prefers to signal through the Gq protein, a subunit of the G-protein that activates phospholipase C. At a molecular level, ligand binding to NTR1 is currently not well understood. The class A GPCRs contain α, β – and γ groups. While the α group usually binds minor endogenous agonists inside the core of the transmembrane, the β group binds agonists of varying sizes. Most GPCR receptor structures belong to the α group of class A, while the NTR1 belongs to a major peptide receptor family in the β group. Even though some structural information is known about the peptide receptors from the γ group of class A GPCRs, structural information about the β group is still unknown (White et al., 2012).

3.3.2 Neurotensin in wound healing

NT acts on immune cells such as dendritic cells, mast cells, macrophages and leukocytes. It stimulates chemotaxis and cytokine release, both of which are needed in order to get a precise immunomodulation response. Furthermore, NT influences new vessel formation, vasoconstriction/vasodilation and vessel permeability, which all assist in improving angiogenesis during wound healing (Moura et al., 2014b).

A study by Moura and colleagues (2014b) tested wound healing in control mice and diabetic mice treated with a NT solution, a collagen dressing and a NT-loaded collagen dressing. PBS was added to non-treated wounds. The study showed no considerable differences among the treatments for control mice. However, major differences were observed in the diabetic mice after day 3. Compared to the non-treated (PBS) wounds, the NT solution had reduced the wound sizes by 11% while the NT-loaded dressing had reduced the wound sizes by 17%. The mice treated with the collagen dressing without NT, had by day 5 reduced the wound sizes by 18% compared to the control wounds (Moura et al., 2014b).

3.4 Nisin

Nisin is an important member of a large class of bacteriocins produced by bacteria that belongs to *Lactococcus* and *Streptococcus* species. They can kill or inhibit the growth of other bacteria. Nisin is widely used as a food preservative in canned products in US to inhibit the growth of bacte-

rium *Clostridium botulinum* (Jones et al., 2005). Since then, many natural and genetically modified variants of nisin have been identified, and studied for their unique antimicrobial properties. Nisin is generally regarded as a safe peptide with recognized potential for clinical use and as potential therapeutic agent for infectious diseases (Shin et al., 2016).

3.4.1 Nisin structure

There are few known variants of nisin: Nisin A, Nisin Z, Nisin F and Nisin Q. Nisin A, the most abundant natural variant of nisin, is a 34 amino acid peptide. Other variants differ from nisin A by one, two and four amino acids respectively. However, all of them have antimicrobial activity against a range of *S. aureus* targets (Shin et al., 2016). The structure of nisin was described by Gross and Moller in 1971. Nisin belongs to a group of cationic antimicrobial peptides collectively called Type A (I) lantibiotics. It contains dehydrated amino acid residues (serine and threonine) and thioether amino acids that form five lanthionine rings, which are characteristics of nisin and lantibioticsv (Shin et al., 2016). These rings are linked by the sulfide bridges between the alanine residues.

3.4.2 Nisin in wound healing

Nisin is both a cationic and amphiphilic peptide. It mediates diverse effects on membrane processes similar to HDPs. Considering the similarity of this peptide to HDPs, it is possible that the immunomodulatory properties associated with HDPs may also apply to nisin (Shin et al., 2016). The efficacy of nisin was investigated by Heunis and co-workers (2013) using an electrospun nanofiber nisin-containing wound dressing, which diffused active nisin onto skin wounds. In a murine excisional skin infection model, the nisin-containing wound dressing significantly reduced the *S. aureus* colonization as analyzed by bioluminescence. In addition, the wounds showed signs of accelerated wound closure of excisional wound (Heunis et al., 2013). Moreover, no adverse effects were observed by histological analysis making nisin even more potent candidate to become an antimicrobial drug.

3.5 Substance P

Substance P (SP) is a neuropeptide and a member of the so called tachykinin family of neurotransmitters, which is one of the largest families of neuropeptides. It is synthesized as a protein and then converted to an active undecapeptide. It has been identified in the peripheral and central nervous system as well as in the intestinal tract (Payan et al., 1984)(Um et al., 2016). SP is an essential factor in mobilization of bone marrow-derived stem cells, thereby endothelial progenitor cells (EPC)

(Um et al., 2016). SP is involved in the wound healing process, in which it is implicated in enhanced cellular proliferation of dermal fibroblasts (Gallagher et al., 2007).

3.5.1 Substance P in wound healing

A study made by Um and colleagues (2016) showed a correlation between SP and wound healing. Different models were used in the study in order to investigate the role of SP (Um et al., 2016). SP signals EPCs to mobilize where needed e.g. into the peripheral blood. They can further be integrated to injury sites as well as angiogenesis. Studies have shown that delayed EPC mobilization can result in chronic wounds (Gallagher et al., 2007). A study performed by Um and colleagues (2016) tested SP treatment on mice. All the mice used in the experiment, treated with SP showed re-epithelialization, which suggests that SP accelerates wound healing. This is demonstrated in Figure 12 (Um et al., 2016).

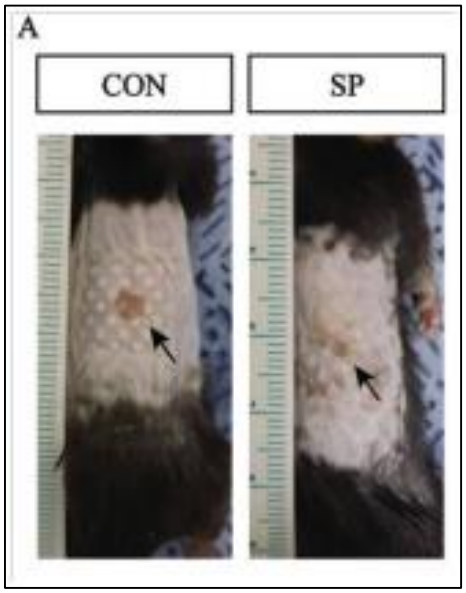


Figure 12 ***SP's effect on wound*** *healing shows a result from Um et al. 2016, where SP shows an enhanced wound healing after 5 days in image (B) in proportion to wounds control in image (A) (Modified from Um et al. 2016).*

A study by Leal and colleagues (2015) investigated the effect of a topically applied SP-containing wound dressing in diabetic rabbit and mouse skin wounds. Furthermore, the study investigated whether a SP deficiency or a deficiency in its receptor, neurokinin-1 receptor, have an impact on wound healing in mouse models. The study showed advanced wound healing, in both rabbits and mice when treated with SP. Additionally, it showed impaired wound healing in mice in the absence of SP or its receptor (Leal et al., 2015).

In a study by Kant and colleagues (2013) topically applied SP was investigated for its temporary effects in regulating multiple cytokines and growth factors involved in cutaneous wound healing in rats. Compared to the control group, daily SP application substantially increased wound closure in open excision wounds. Furthermore, treating the wounds with SP, substantially decreased IL-10 levels and increased TNF-α levels on day 3. Contrarily, the level of IL-10 increased and TNF-α decreased on day 7. In the SP-treated wounds, the protein and mRNA expressions of TGF-β_1 and VEGF both increased on day 3 and day 7, and decreased on day 14. The results of this study demonstrated that SP-treated wound healing was advanced by regulating both growth factors and cytokines. This suggests that SP could be a promising novel therapeutic agent in diabetic wounds where SP, TGF-β_1 and VEGF levels all decrease with the impaired inflammatory reaction (Kant et al., 2013).

Several studies have shown contradicting results when investigating the activity of SP in wound diseases here among psoriasis. According to a study performed by Maggi (1997), the release of SP can initiate degranulation of mast cells contributing to an early inflammatory cascade in psoriasis (Maggi, 1997). Furthermore a study performed by Zaki and colleagues (2010) reported that SP levels in patients diagnosed with psoriasis are substantially higher than SP levels in the control group. Additionally, the study showed increased SP plasma levels and, mRNA expression. This suggests that SP play a substantial role in psoriasis (Zaki et al., 2011).

4.0 Methods

In this section, protocols behind the main used methods are described to clarify the functions of the components and the experimental procedures. For better understanding of the procedure, schematic drawings are inserted. Detailed protocols can be found in the appendix, 1.0 Experimental procedure.

4.1 Subcultivating the cell line

The keratinocyte cell line used in this project, HaCaT, was grown in Dulbecco's Modified Eagle's Media (DMEM) with the addition of GlutaMAX and 10% Fetal bovine serum (FBS) in a T75 flask surface in a 37ºC, 5% CO_2 incubator. GlutaMAX contains L-glutamine, which prevents ammonia build-up during long-term cultures (Thermo Fisher, 2015) and FBS is a serum supplement, which is rich in growth factors (Biological industries, 2016). The CO_2 is ensuring a neutral, stable pH in the culture. The HaCaT cell line was subcultivated twice a week depending on the confluence, by a standard method using trypsin as a detaching agent. Before detachment, the culture media was removed from the flask and the cells were washed twice with phosphate-buffered saline (PBS) to get rid of leftover residues, which could deactivate the trypsin. 2 mL trypsin was added to the flask and incubated for 10 minutes. Next, 8mL DMEM media with 10% FBS were added and the culture was gently resuspended in order to mono up the lumps of cells. 10µL cell culture were then placed on the hemocytometer and the cells were counted. Lastly, 1 million cells were transferred to a new flask to enable further propagation and placed back in 37ºC incubator. This was done every three to four days.

4.2 Hemocytometer

The hemocytometer is used to count the cells in a culture. ~10µL of the culture is added under the microscopic slide and the hemocytometer is inspected under the microscope. The colonies within the gridded area (see Figure 13 below) are counted with a click counter and calculated back to obtain the concentration of the cell culture (Bastidas, 2016).

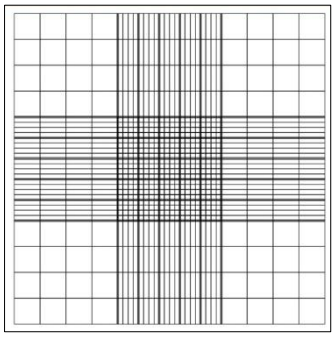


Figure 13 ***Grid of the hemocytometer*** (Cell Counting with Neubauer Chamber, Basic Hemocytometer Usage, 2016).

The following formula is used to calculate the concentration:

$$Concentration\ (cells/mL) = \frac{sum\ of\ counted\ colonies \times 10.000}{volume\ (in\ mL)}$$ (Bastidas).

4.3 Scratch assay

The scratch assay is performed *in vitro* and is a well-developed, convenient and easy method to measure cell migration. The scratch assay mimics the migration of cells throughout the *in vivo* wound healing process and is specifically appropriate when studying the impact of cell-cell interactions and cell-matrix interactions on cell migration (Liang et al., 2007).

Before the cells were transferred, the 48 well plate was prepared. As seen in Figure 14 below. Horizontal lines were drawn from A1-4 to D1-4 in order to have a measure point for measurements and pictures. Before starting the scratch assay, the cells were seeded and incubated overnight. 65.000 cells were added to each well along with 250µL media. Untreated cells were used as a control.

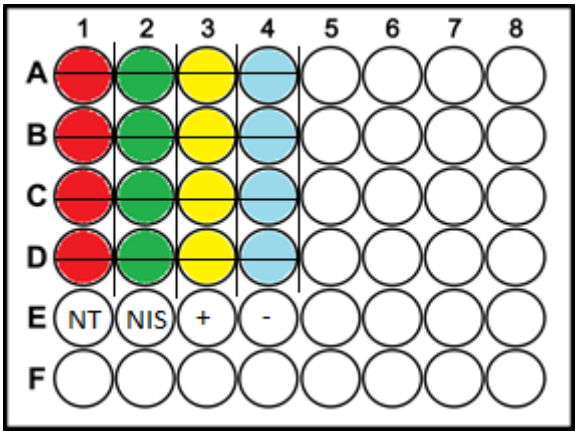


Figure 14 ***A 48 well plate.*** *shows a 48 well plate. The colors indicate that they contain the same solutions. Line E explains what is placed in row 1 through 4. The horizontal lines indicate a starting point for measurements and pictures. 65.000 cells were added to each well along with 250µl media. The total peptide concentration in a well should be 25µg/ml*

The cells' confluency had to be at least ~70% before the assay was started. In each well, a scratch was made with a yellow pipette tip, the media was removed and the cells were washed twice with 250µL PBS. Next, the stimuli were added to the wells, one with neurotensin and 2% FBS media and one with nisin and 2% FBS. The total peptide concentration in a well was 25µg/mL. 250µL peptide stimuli were added to each well. The scratch width was measured with a special measuring ocular and pictures of the wounds were taken. The observations were made 0, 4, 7 and 18 hours after stimulation. Figure 15 shows the scratch made in each well.

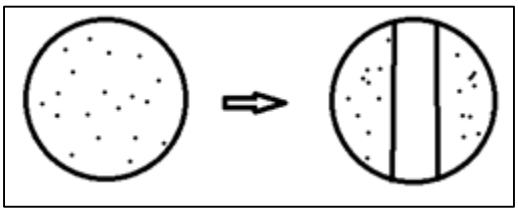


Figure 15 ***Scratch illustration.*** *illustrates a well before and after a scratch*

4.4 Cell harvest

The cell harvest was performed by adding TRI reagent to the wells. The media was removed and the cells were washed twice with 250µL room temperature PBS. Next, the cells were harvested by adding 2x 100µL TRI reagent to each well and resuspended. The cells were then transferred to Eppendorf tubes. The samples were placed in a -80ºC freezer until the RNA isolation. TRI Reagent

contains a mixture of guanidine thiocyanate and phenol, which effectively dissolves DNA, RNA, and protein on lysis of tissue sample (Sigma-Aldrich Co, 2016).

4.5 RNA isolation

RNA isolation is started by thawing the harvested cell samples. 0.2mL chloroform were added for each mL TRI reagent and the tubes were vortexed for 15 seconds. Chloroform removes the contaminating proteins by partitioning in the lower phase of the two-phase solution (Reed et al., 2007). The samples were left in room temperature for 10 minutes. Throughout the 10 minutes, layers appeared in the sample tubes and new 1.5mL eppendorf tubes were prepared. After 10 minutes, the samples were placed in the centrifuge (4ºC, 12000g) for 10 min. When taken out, three layers were completely clear, a bottom pink layer of protein, a middle white layer of DNA and a top clear layer of RNA. The top layer was carefully transferred to new tubes and left in room temperature for 10 minutes. Next, 0.5mL isopropanol were added to the tubes for each mL TRI reagent to precipitate the RNA. The tubes were vortexed and placed in the centrifuge (4ºC, 12000g) for 10 minutes. The supernatant was discarded and each sample was washed with 200µL 75% ethanol, centrifuged at 4ºC, 7500g for 5 min and the supernatant was discarded again. The pellets containing RNA were left to air-dry for 10-15 minutes, depending on how much fluid there was left. The washed pellets were resuspended in ~30µL of ddH_2O. To measure the isolated RNA concentration, a Nanodrop machine was used. The wavelength was set to 260nm as RNA (and DNA) has a maximum absorption at this wavelength. This corresponds to ultraviolet light (Reed et al., 2007). Moreover, the ratios between wavelengths 260 and 280nm as well as 260 and 230nm were measured on Nanodrop. The ideal RNA values for both ratios should be ~2 as that indicates pure RNA. Lower ratios indicate contamination (Thermo Fisher Scientific, n.d.).

4.6 cDNA synthesis

complementary DNA (cDNA) is produced from isolated mRNA using reverse transcriptase (RT). RT is an enzyme that catalyzes RNA-directed DNA synthesis. Apart from the enzyme, which is required for the reaction to occur, RT buffer, random primers, dNTP mix, ddH_2O and RNA are also present. Buffers that contain e.g. $Mg2^+$, helps to obtain higher yield of the reaction. Dithiothreitol is a reducing agent that breaks the bonds of secondary RNA structure and Tris-HCl ensures stable pH during the reaction. The dNTP mix is a mixture of all four nucleotides (adenine, thymine, cytosine, guanine), which becomes incorporated into the cDNA. Under normal conditions, all nucleotides are present in the mix in equal concentrations (Reed et al., 2007). Random primers are added to the

mix, which bind to mRNA and this, synthesizes the cDNA. The next step is the polymerase chain reaction (PCR), which is used to amplify the cDNA. In this step house keeping primer (RPLP0) is added to the cDNA.

4.7 The PCR reaction

PCR is a rapid, inexpensive and simple way of producing significant amounts of DNA that are large enough for analysis. The machine used for PCR is called a thermal cycler and can be set to run the reaction with varied temperature, times and number of cycles (Tortora et al., 2010). A single PCR cycle consist of three steps, which are carried out at different temperatures: denaturation of double stranded DNA (dsDNA), annealing of the primers and extension of the primers by DNA polymerase. Denaturation of dsDNA happens at temperatures 94-98°C. Strands are separated into single strands of target DNA (ssDNA). Annealing of the primers occur at temperatures ranging from 37-65°C depending on the primer composition. Extension of the primers is done at 72°C (Reed et al., 2007).

4.8 Gel electrophoresis

Gel electrophoresis is a widely-used method for separation of nucleic acids. Electrophoresis is a term used to describe the movement of ions in an applied electrical field. The molecules of DNA are negatively charged (from phosphodiester bonds in the backbone structure) and therefore migrate towards anode in the gel box. The gel was made of 1g agarose powder (1%) and 100mL TBE buffer. 10µL ethidium bromide (EtBr), a fluorescence tag, was added to the liquid agarose and to the running buffer. EtBr is carcinogenic and should be handled carefully. The well combs were placed in the gel tray and the agarose was then poured into the gel tray and left to solidify for 30-45 minutes. Once solidified, the tray was placed in the gel box. All samples were mixed with loading buffer before loaded onto the gel. A ladder mixed was loaded into the first and last well as a guide to interpret the band sizes (in kb). Next, the PCR products were loaded into the wells, first the samples with RT and next the samples without. The samples were mixed with loading buffer to ensure that migration can be followed visually. After the gel was run (time and volt depend on the expected result), it was removed from the gel box and placed in an UV device to visualize the DNA fragments and a picture was taken (Reed et al., 2007).

4.9 Real time PCR

Real time PCR (qPCR) is a variation of standard PCR. In qPCR, it is possible to simultaneously amplify and quantify template DNA in a sample by establishing the number of copies present by

working in the exponential phase of amplification. The outcome of qPCR is visual because of the fluorescent SYBR Green dye. The level of the fluorescence is monitored during the reaction. Any increase in fluorescence is directly proportional to the amount of dsDNA produced as SYBR Green dye binds strongly to dsDNA (enhancement in fluorescence of 100-fold). In order to read the results from the samples, a standard curve needs to be run in duplicates prior to the analysis (Reed et al., 2007).

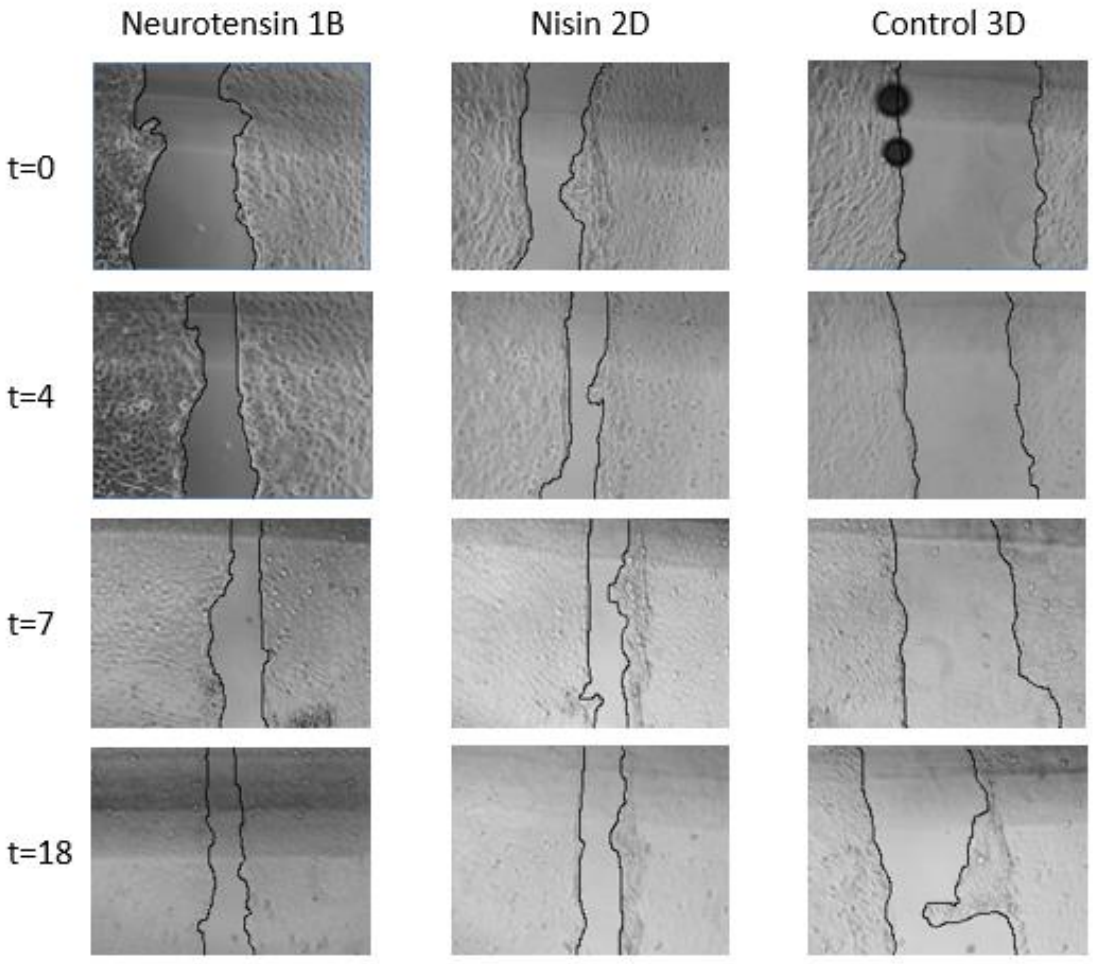
5.0 Results

In this section, results from the experimental procedures are presented, including scratch assay and RNA isolation. Furthermore subsequent processing of the results is also presented with examples.

5.1 Scratch assay

Four replicates were made for each sample: neurotensin (A-D), nisin (A-D) and control (A-D). The images below represent one of the four replicates of each sample at time (hours) 0, 4, 7 and 18. They were taken by a microscopic camera. Note that the image of the control at time 0 has black spots. Those are air bubbles, which do not affect the results. Images of the samples not presented in this section can be found in the appendix, section 2.0 Results.

5.1.1 Wound images



The scratches show a distinct reduction in size from t=0 to t=4. At t=7 and t=18 little to no difference is seen.

5.1.2 Wound measurements

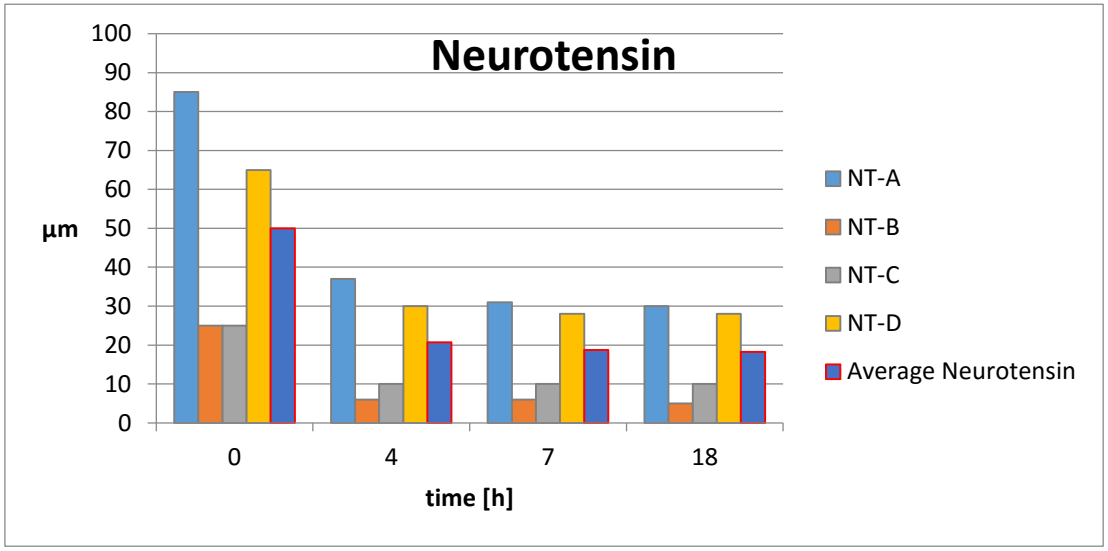
Wound measurements are shown in Table 2 below. All wound size results are measured in µm.

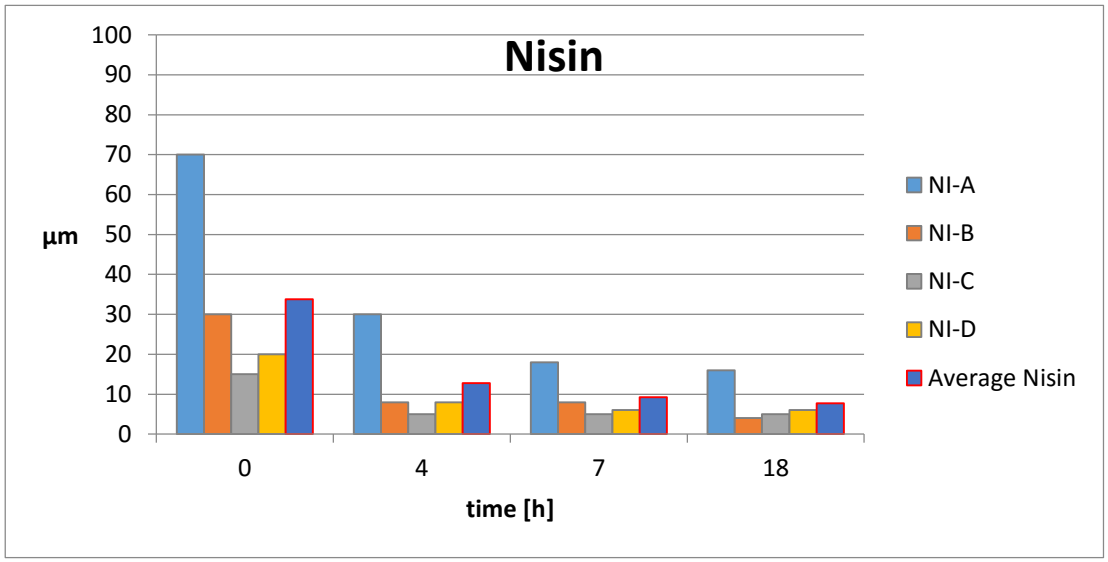
Table 2 shows the measurements of the wound sizes at hours: 0, 4, 7 and 18. The sizes of the wounds are measured in µm.

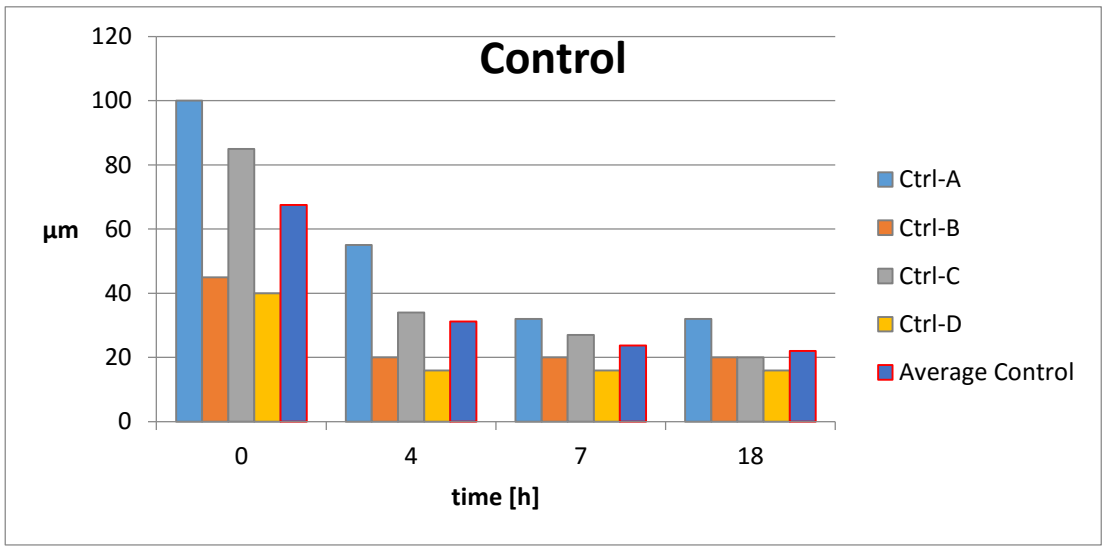
	Neurotensin	**Nisin**	**Control**
t=0			
A	85	70	100
B	25	30	45
C	25	15	85
D	65	20	40
t=4			
A	37	30	55
B	6	8	20
C	10	5	34
D	30	8	16
t=7			
A	31	18	32
B	6	8	20
C	10	5	27
D	28	6	16
t=18			
A	30	16	32
B	5	4	20
C	10	5	20
D	28	6	16

5.1.3 Wound size graphs

The following graphs below show the sizes of the wounds at different times (h). The graphs show the four replicates of neurotensin in the first graph, nisin in the second and control in the third. Time (hours) is plotted on the x-axis and size (µm) on the y-axis.


Neurotensin
100
90
80
70
60
µm 50
40
30
20
10
0
0
4
7
18
time [h]
NT-A
NT-B
NT-C
NT-D
Average Neurotensin


Nisin
100
90
80
70
60
µm 50
40
30
20
10
0
0
4
7
18
time [h]
NI-A
NI-B
NI-C
NI-D
Average Nisin


Control
120
100
80
µm 60
40
20
0
0
4
7
18
time [h]
Ctrl-A
Ctrl-B
Ctrl-C
Ctrl-D
Average Control

5.1.4 Subsequent processing of results

The average scratch closure of neurotensin, nisin and control were calculated for time 0, 4, 7 and 18 hours. The neurotensin calculations are seen below. See appendix 2.0 Results, for the calculations of nisin and control.

5.1.4.1 Neurotensin

Table 3 shows the scratch sizes of the four replicates of neurotensin at time 0, 4, 7 and 18. The wounds are measured in µm.

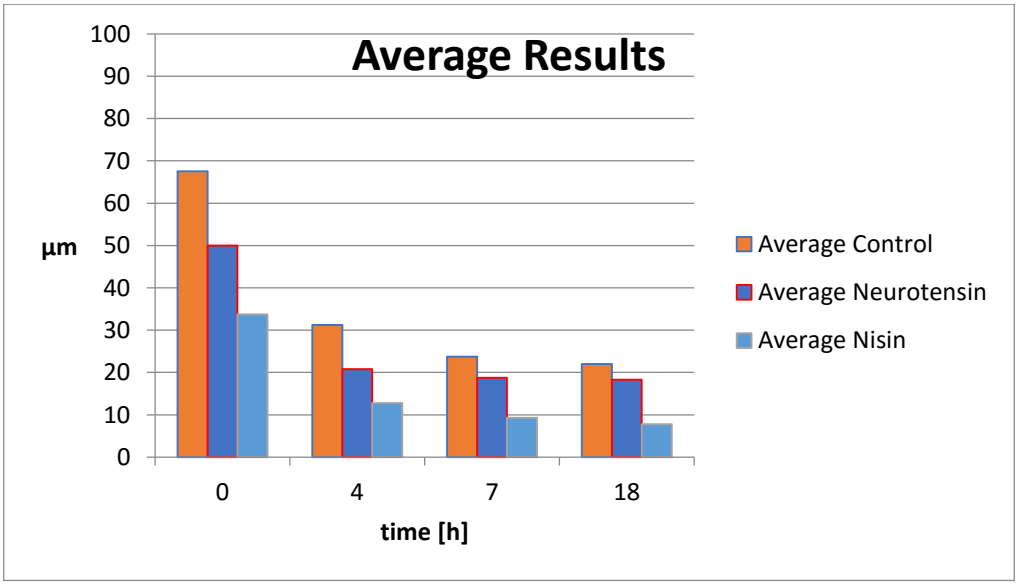
	t=0	t=4	t=7	t=18
A	85	37	31	30
B	25	6	6	5
C	25	10	10	10
D	65	30	28	28

Table 4 shows the average scratch size of the four neurotensin replicates measured in µm.

t=0	t=4	t=7	t=18
50	20.75	18.75	18.25

5.1.4.2 Calculating the average results of the four replicates of neurotensin, nisin and the control

The average scratch sizes for neurotensin, nisin and the control are all shown in the graph below. This was done to get a better visual of the average sizes and to determine the possible effects of the two peptides. Time (hours) is plotted on the x-axis and size (µm) on the y-axis.



The scratch closure percentage were calculated using the following formula:

$$\left(1 - \frac{t(18)}{t(0)}\right) * 100\% = \text{scratch closure in percentage.}$$

The calculation of neurotensin sample A's closure in percentage is shown below. See appendix, section 2.0 Results for the calculations of neurotensin B, C, D, nisin (A-D) and control (A-D).

$$\left(1 - \frac{30\ \mu m}{85 \mu m}\right) * 100\% = 64.7\ \%$$

The average wound closure was calculated in percentage for neurotensin, nisin and the control. They are shown in below.

Table 5 shows the average scratch closure in percent of neurotensin, nisin and the control.

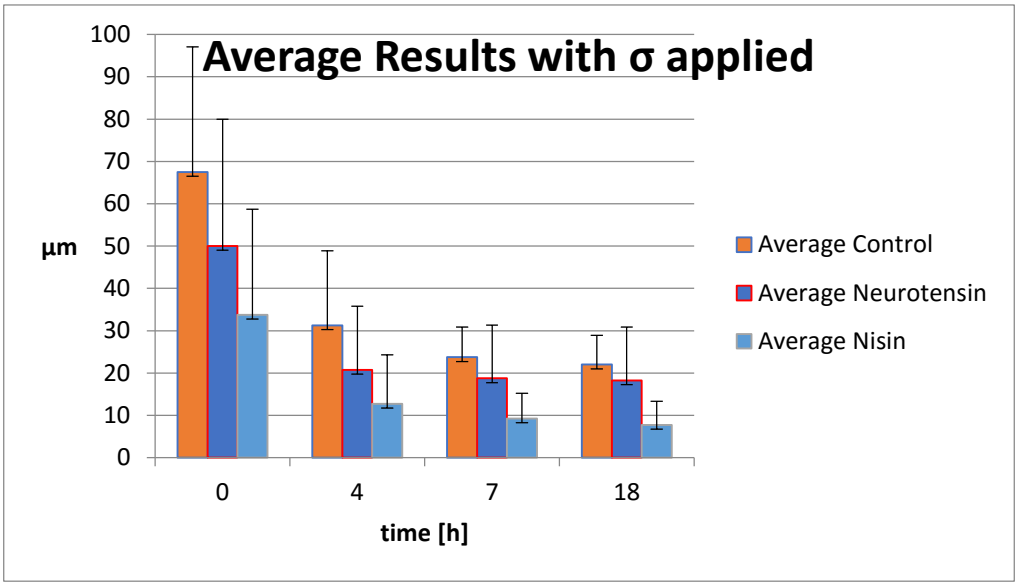
Neurotensin	**Nisin**	**Control**
65.4%	75.1%	65.0%

The standard deviation of neurotensin, nisin and control at time 0, 4, 7 and 18 hours, as well as, total wound closure was calculated using Excel and is shown in Table 6.

Table 6 shows the standard deviation of neurotensin, nisin and control

time (h)	**σ** NT	**σ** NI	**σ** Ctrl
0	30.0	24.9	29.6
4	15.1	11.6	17.6
7	12.6	5.9	7.1
18	12.6	5.6	6.9

The average wound closure results with standard deviation (σ) applied can be seen in the graph below. On the x-axis, time (hours) is plotted while size (μm) is plotted on the y-axis.



The relative standard deviation is then calculated using following formula:

$$\%RSD = \frac{standard\ deviation}{average} * 100\%$$

For average scratch sizes, see appendix section 2.0 Results.

An example for neurotensin at t=0 is shown below:

$$\%RSD = \frac{standard\ deviation}{average} * 100\% = \frac{30}{50} * 100\% = 60\%$$

Relative standard deviations for neurotensin, nisin and control at time (hours) 0, 4, 7 and 18, as well as, total scratch closure was calculated using Excel and is shown in Table 7.

Table 7 shows the relative standard deviation for both neurotensin, nisin and control at time (hours) 0, 4,7 and 18.

time (h)	**Relative standard deviation (RSD)**		
	RSD $_{NT}$	**RSD $_{Ni}$**	**RSD$_{Ctrl}$**
0	60.0%	73.9%	43.8%
4	72.7%	90.9%	56.4%
7	67.1%	64.5%	30.0%
18	69.1%	71.8%	31.5%

5.2 RNA isolation

Table 8 below shows the results from the RNA isolation.

Table 8 shows the results from the RNA measurements.

	ng/µl	**260/280**
NT-1A	189.3	1.77
NT-1B	572.2	1.56
NT-1C	133.7	1.80
NT-1D	243.7	1.96
NI-2A	0.2	-0.38
NI-2B	134.5	1.72
NI-2C	12.6	1.92
NI-2D	48.5	1.80
Ctrl-3A	131.2	1.52
Ctrl-3B	43.1	1.48
Ctrl-3C	432.7	2.19
Ctrl-3D	42.4	1.78

5.3 Gel electrophoresis

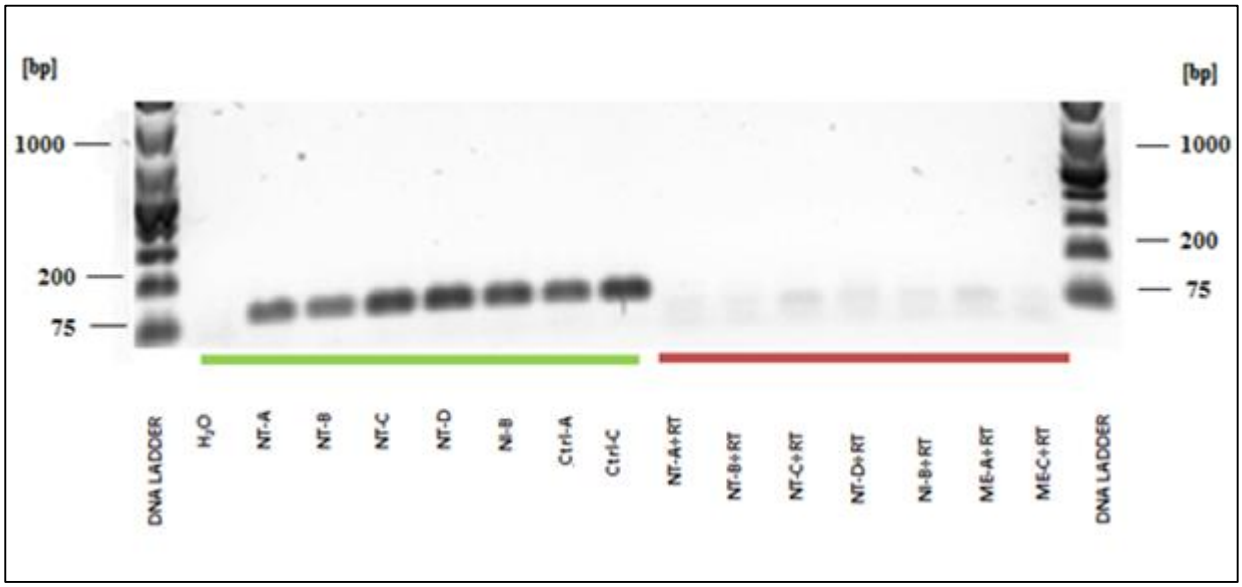


Figure 16 shows the results from the electrophoresis. The green part shows the samples from right H_2O, neurotensin A-D, nisin –B and control A+C with the enzyme reverse transcriptase (RT). The red part shows the samples neurotensin A-D, nisin –B and control A+C without the enzyme RT. The DNA ladder used as marker is 1 kb.

6.0 Discussion

The use of antibiotics as therapeutic agents against many infections caused by pathogens is a widely accepted treatment. Many antibiotics are helpful in fighting infections. However, pathogens are developing resistance against multiple antibiotic strains. The threat that all pathogens eventually will develop antibiotic resistance, has presented a need for alternative ways to treat the infections. HDPs are of particular interest, as they are found in the human body, mainly in various epithelial surfaces, in substantial quantities and are shown to be active against bacteria, viruses and fungi .The role of HDPs should not be underestimated. Within the host, they have the ability to create anti-inflammatory responses to pathogen entry and infections. Additionally, HDPs can target multiple processes in bacteria essential for their survival. These processes include e.g. utilization of cell wall peptidoglycan and inhibition of RNA, DNA and protein synthesis. Due to this variety of antimicrobial mechanism targets, HDPs are less likely to develop resistant strains (Mansour et al., 2014). This is important as the risk of bacteria obtaining the resistance against HDPs is significantly lower and makes those peptides more desired therapeutic agents than already known antibiotics (Wang, 2014). If research on these peptides shows promising results, HDPs could be an alternative treatment to skin diseases and wound healing. Therefore, the aim of this project was to investigate the immune modulating role of four chosen HDPs, insulin, NT, nisin and SP, in regard to cytokine regulation in a wound healing model. In injury and infection of the skin, expression of HDPs is upregulated due to increased synthesis by keratinocytes and deposition from degranulation of recruited neutrophils. This leads to the conclusion that, according to the literature, HDPs play an essential role in the wound healing.

6.1 Peptides of our interest and current research

In general, HPDs, as mentioned above, have a pivotal role in wound healing. However, to specify the discussion and the aim of this project, four peptides were chosen for closer investigation. During the course of this project, few new articles describing promising research on the topic of the role of HDPs in wound healing were published.

Insulin is a hormone responsible for lowering blood sugar levels, which has antimicrobial activities. It has been reported that application of insulin has an acceleration of wounds closure in skin burn rats and advanced healing from incision wounds in rabbits. In humans, this hormone is helpful in the treatment of diabetic ulcers (Liu et al., 2009b)(Chen et al., 2012). Moreover, it has been shown that topical application of insulin to skin wounds in mice, stimulates a regenerative process in the

wound tissue and re-epithelialization. Insulin interacts with its receptor and affects keratinocyte behavior (Liu et al., 2009b). The before-mentioned studies are supported by the study performed by Zhang and Lv (2015), in which the role of insulin in wound healing in patients with diabetic foot ulcers was investigated (Zhang & Lv, 2015). In this study, a group of patients were injected with biosynthetic human insulin, into the base of the diabetic foot ulcer twice a day, for 7 days. It was proved that local injection of insulin was more effective than application of dressings due to its maintenance of high concentration and long efficacy duration. Moreover, injections of insulin resulted in lower blood glucose levels in patients, which may promote wound healing by improving the growth of granulation tissue. However, no evidence indicated that the peptide accelerated wound healing (Zhang & Lv, 2015). These findings, confirm that the use of insulin in the wound healing process is helpful, not only in re-epithelialization but also in the underlying granulation tissue and it can become a potential treatment. Even though multiple studies are attempting to elucidate the role of insulin in wound healing, the mechanisms are not fully understood and need further investigation.

Substance P is a neuropeptide. A correlation between SP and wound healing has been shown in a study by Um and colleagues (2016) by enhancing angiogenesis. The results showed that an injection of SP led to the acceleration of wound healing in skin through a better reconstitution of blood vessels. It is also suggested that EPC regenerates the endothelial lining of blood vessels (Um et al., 2016) Another study performed by Leal and colleagues (2015) tested a topical wound dressing containing SP in diabetic mice and rabbit skin wounds. The study showed accelerated wound healing in both mice and rabbits (Leal et al., 2015). Additionally, a study by Kant and colleagues (2013) investigated the effect of topical applied SP in correlation with cytokine regulation in cutaneous wounds in rats. The study showed that SP decreased the level of IL-10 while it increased TNF-α levels on day 3. In contrast the opposite was observed on day 7 (Kant et al., 2013). This suggests that SP accelerates wound healing by regulating cytokines. Furthermore, SP has been shown to play a crucial role in skin diseases as e.g. psoriasis. This was demonstrated in a study by Maggi (1997) in which SP release was shown to initiate mast cell degranulation, which contributes to early inflammation (Maggi, 1997). Zaki and colleagues (2010) showed that SP levels in psoriasis patients are relatively higher than the control group (Zaki et al., 2011). Both of these studies support the role of SP in psoriasis.

Other studies, one made by Park and colleagues (2016) and one by Zhu and colleagues (2016) confirm the findings of the studies performed by Um and colleagues (2016), the study by Kant and col-

leagues (2013) as well as the study made by Leal and colleagues (2015). SP was found to promote wound healing of diabetic ulcers through suppression of inflammation, induction of angiogenesis and mobilization of stem cells (Park et al., 2016). In a study by Park and colleagues (2016) the mice with ulcers, as well as a control group of mice, were subjected to intravenous injection of SP twice a week for 2 weeks. The SP-treated mice exhibited accelerated wound closure (on day 7 post-wounding), no severe inflammation, decreased TNF-α levels and increased level of IL-10. Moreover, after 2 weeks of treatment, mice within the SP-treated group showed a fully covered epithelial layer and more granulation tissue with increased vessel formation. Further, the stem cells level in bone marrow, which is very low in diabetes was restored to normal levels by SP (Park et al., 2016). In another study by Zhu and colleagues (2016) rats with diabetic wounds were subjected to injection treatment with SP (Zhu et al., 2016). The study showed that SP combined with epidermal stem cells has a positive effect on multiple processes in wound healing like e.g. promotion of fibroblast proliferation, cell migration and differentiation, and acceleration of blood vessel and granulation tissue formation. It was also noted that this treatment provides a good environment for epidermal cell proliferation and migration (Zhu et al., 2016). The results of these studies all support SP as a potential new therapeutic agent that advances e.g. angiogenesis and fibroblasts proliferation all of which accelerates wound healing.

NT regulates multiple biological processes, like e.g. intestinal epithelial cell proliferation. It interacts with mast cells, leukocytes and macrophages, which induces cytokine release and enhances chemotaxis (de Silva et al., 2011). NT influences processes like vessel formation, which assist in improving angiogenesis during wound healing. It has been reported that NT can advance diabetic wound healing and that its activity can be further enhanced when it is applied in collagen based dressings in mice (Moura et al., 2014b). Similar experiments were performed on the hyperglycemic human keratinocytes, mimicking the diabetic foot ulcer cells, and under those circumstances, NT did not affect proliferation nor expression of the tested inflammatory cytokines and growth factors e.g. TNF-α, IL-1β and IL-6 (Moura et al., 2014a). This shows that the processes, which has led to successful wound healing in mice, do not correspond with the *in vitro* processes in human cells. It also shows the complexity of the wound healing process and the fact that multiple experiments need to be performed to obtain reliable results, which could confirm that the use of NT truly aids the wound healing process.

Nisin is a bacteriocin that kills or inhibits the growth of bacteria. Nisin has been shown to mediate diverse effects on membrane processes similar to HDPs (Shin et al., 2016). In an experiment conducted on mice, wound dressings containing nisin were used. The colonization of *S. aureus* was significantly reduced by the treatment, showing accelerated wound healing (Heunis et al., 2013). However, this study is the only one showing the above mentioned effect. A probable reason for that is the fact that nisin is a relatively new peptide in wound healing research.

Neurotensin and nisin were both investigated experimentally, the results are discussed below.

The measurements of the wound sizes showed gradual cell migration to close the wound. The average percentages for NT, nisin and control are 65.4%, 75.1% and 65.0%, respectively. This shows that even without the added stimuli, there is a considerable migration of cells. Comparing the result of the control with the result of the NT stimulation, nearly no difference is seen, which leads to the conclusion that, in this scratch assay, NT did not have any stimulatory effect in the wound healing process. The average percentage of wound closure for nisin is, on the contrary, higher than in the unstimulated cells. This result could indicate a stimulating role of nisin in wound healing. However, due to the time constraints, only one full scratch assay was performed, and no conclusion can be drawn from the data. Moreover, standard deviations are relatively high for all replicates. It is a common practice that triplicates are required to obtain reliable experimental results. Nevertheless, stimulation of the cells with nisin, gave the most promising results, a tendency that needs to be further investigated to be confirmed. The true correlation of the peptide stimulations and aided wound healing can only be established from cytokine expression by qPCR. Additionally, the scratch assay was not performed with the other two peptides due to time constraints. There are however, some studies, which have shown accelerated wound healing in different model organisms while applying one of the peptides.

6.2 Analysis of the experimental process

Several experiments (see appendix section 1.0 Experimental procedure) were performed in order to fulfill the aim of the project. Few problems occurred while growing the HaCaT cell culture. First, the media supplied at the beginning of the experiment was DMEM media with low glucose concentration. The cells were growing poorly in this media and were reaching 1 million cells/mL in a week while at least twice as much would be expected. Moreover, due to contamination in the suction tube, the cells were found unviable. New cells were subcultivated in the cell lab and the media was

changed to the high glucose DMEM. After this change, the cell growth returned to, what could be called normal, and subcultivations were performed every 3-4 days.

Two scratch assays were performed throughout this project. In both assays, NT and nisin were simultaneously tested. Only the results from the second scratch are presented in the 5.0 Results section. The first assay was started following the procedure in the 4.0 Methods section, however, the cells already stopped migrating 7 hours after stimulation. The initial reason, given by professor Håvard Jenssen (personal communication), was that the FBS concentration should have been 10% while only 2% was used. This reason was disclaimed though, as with such relatively high concentration of growth factors, the closure of the wound would have been caused not only by the migration due to the stimuli but also due to normal growth of the cells. 24 hours later, the cells were observed to be unviable and were therefore discarded. The reason for the death of the cells remained unsolved. The second scratch assay was fully performed. However, due to time constraints, the cells were harvested 18 hours after stimulation with peptides. The harvest of the cells should have been performed after complete wound closure so the speed of the wound closure could be calculated. Due to the fact, that the wounds were not fully closed when harvested, the only calculation that could be performed was the percentage of the wound closure until the time of harvest along with standard deviation.

RNA isolation was performed in two batches, first for all the A and B samples, and second for the C and D samples. The optimal amount of RNA, which should be used to perform a successful cDNA synthesis is 2μg. However, as shown in the Table 8 in section 5.0 Results, samples 1C, 2B, 3A and 3C had a lower concentrations and therefore 10μL of the samples were used for cDNA synthesis. Samples 2C, 2D, 3B and 3D were not used due to substantially lower concentrations. Sample 2A showed negative wavelengths ratios while measuring the concentration in Nanodrop and was therefore also excluded from further analysis. It is worth mentioning, that the low yields of obtained RNA probably are due to lack of experience from the group in performing such procedure. The protocol for RNA isolation has few complicated steps in which it is easy to lose the fraction of interest. The first problem is encountered after chloroform addition. Three phases are visible in each tube, however, only the top layer should be collected. Due to the small operating volumes, it requires precision to gather as much of the upper phase possible without touching the middle phase. This middle phase is DNA which, while collected with RNA, is considered contamination and results in a lower 260/280 ratio. The 260/280 ratio should be ~2 for pure RNA samples. Some of the samples (2A, 3A, 3B) have a 260/280 ratio substantially lower than 2 implying contamination.

Moreover, in the next step in which isopropanol is added and the tubes are centrifuged, the pellet with RNA is transparent and therefore cannot be seen. Only by centrifuging the tubes with the cap facing the inner side of the rotor, is it possible to assume, that the pellet is situated on the opposite site of the tube and the supernatant can be carefully discarded. However, also in this step some RNA can be lost due to the discard of the pellet with supernatant. The last step is air-drying of the samples after they have been washed with ethanol.

The cDNA synthesis was performed on samples 1A-D, 2B, 3A and 3C. Later, the PCR with hot start master mix was performed on these samples. Next, gel electrophoresis was performed to visualize the bands and evaluate the success of cDNA synthesis. A sample with water instead of template was used as a control. The gel showed a clear band in the wells, in which the samples with RT were loaded. The size of the bands, when compared to the sizes of the marker, is between 75 and 200bp. This is the expected size. The samples without RT showed vague bands, which is a desired result as no cDNA can be produced without this enzyme. To sum up, the results obtained on the gel show that the cDNA synthesis was successful and the samples contain the cDNA. Moreover, the intensity of all the bands is nearly the same indicating similar amounts of cDNA produced. Those samples could be used for the further analysis.

Unfortunately, due to time constraints and technical problems, the crucial part of this project, the quantification of cytokine gene expression in the keratinocytes treated with peptides, was not performed. In order to get the results from the qPCR method, a standard curve needs to be run prior to proper analysis of the samples. As the levels of the IL-6, -8 and -10 were too low in untreated keratinocytes to prepare a standard curve, this step could not be performed. Therefore, running the analysis on the samples alone was a waste of time and material as comparing them to standards was not possible. Comparison of the samples with standards would show whether the cytokine level is up- or down-regulated in the samples.

7.0 Conclusion

Setting out to find the effect of HDPs on wound healing turned out to be ambitious in regard to our time constraints. When starting the project, the aim was to establish the immune modulating role of 4 peptides: insulin, NT, nisin and SP in regard to cytokine regulation in keratinocytes, and compare the findings to literature research.

Studies of insulin shows that its antimicrobial activity advances wound healing, particularly in diabetic subjects. These results indicate that insulin is a promising candidate as a potential therapeutic agent. Further *in vivo* studies are needed in order to confirm its abilities.

SP studies shows that it is capable of advancing angiogenesis and that SP treated ulcers in diabetic mice displayed an accelerated wound closure and decrease/ increased IL-10 levels. Furthermore SP has been found to be correlated with EPC, as SP signals the cells to mobilize where needed as e.g. in angiogenesis. Additionally, SP levels are high in psoriasis. These results suggest SP to be a potential candidate for novel therapeutic agents.

As NT and nisin were investigated experimentally, the average wound closure percentage could be calculated. We obtained the results of NT's wound closure percentage to be 65.4%, whereas nisin's wound closure percentage was 75.1%. These results were compared to the control, in which the wound closure average was 65%. These findings indicated that nisin in contrast to NT may have a stimulatory effect on wound healing. The literature on these two peptides both support the theory that they play a role in wound healing with potential to become medical treatments.

In order to draw any reliable conclusion from the results, the experiments should be repeated multiple times. As we performed the experiment only once on two out of four peptides, no conclusion can be made. There is, however, potential in the obtained nisin results as the cell migration was advanced when compared to the control.

8.0 Perspective

As previously mentioned, the HDPs' effects on wound healing have been shown by several studies (Liu et al., 2009b, Chen et al., 2012, Zhang and Lv, 2016, Um 2016, Park et al. 2016, Zhu et al. 2016, Moura et al. 2014a, Moura et al. 2014b and Heunis et al, 2013). They all support the theory that insulin, NT, nisin and SP affect the different processes in wound healing as e.g. re-epithelialization, angiogenesis, fibroblast proliferation, cell migration, collagen synthesis and cytokines.

A subject of future studies could therefore include further experimental investigations with focus on the above mentioned processes and how the antimicrobial activity of HDPs affect them. These studies could be performed by repeated scratch assays on all four peptides in order to obtain reliable results. Furthermore, qPCR should be done to analyze the cytokine levels and the stimulatory effect of the peptides.

If more time is available, a standard curve should be made. The standard curve is made by plotting the concentration of each standard on the x-axis against the Ct value for every standard on the y-axis. The ideal standard curve has a slope value of -3.32, which corresponds to a 100% PCR efficiency. The standard curve is made this way in order to obtain the DNA concentration in the unknown samples. To obtain reproducible and precise expression profiling of the genes of interest, reference genes' expression products are used. These are used in order to normalize the expression levels in between experiments e.g. to compare the Ct values with other cell types as different cell types exhibit high and low cytokine expression.

Apart from the previously mentioned, future experiments could include angiogenesis assays, different cytokines and toxicity tests in order to prepare for in vivo human testing.

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Glossary

Angiogenesis: The process of new blood vessels forming from pre-existing vessels;

Annihilation: Complete destruction;

Antigens: Molecules capable of inducing the immune response, namely production of antibodies against the specific antigen;

Antibodies: Also called immunoglobulins; proteins produced mainly by plasma cells in response to pathogens (bacteria, viruses) entering the host; Antibodies recognize antigens;

Bacteriocins: Toxins produced by bacteria to inhibit the growth of similar strains;

Beta-lactam ring: Beta- lactamases are the known hydrolytic amidases that cleave the beta-lactam ring of both penicillin and cephalosporin antibiotics;

Cathelicidins: One of the two major groups of AMPs; Cathelicidins have an α-helix structure; gene located on chromosome 3 and encodes for LL-37;

Cationic amphipathic molecules: Molecules that both have hydrophilic and lipophilic properties. When the lipophilic group is positively charged, the molecule is called a cationic amphipatic molecule;

Chemotaxis: Movement in response to the presence of a chemical;

Collagen: A fibrous family of proteins;

Corneocytes: Flattened dead keratinocytes;

Cytokines: Glycoproteins that regulate the intensity and duration of immune responses;

Defensins: One of two major groups of AMPs; Defensins have a β-sheet structure;

Desmosomes: Cell adhesion proteins and linking proteins;

Discovery void: Most antibiotics were discovered between 1945 and 1987 and since then no new antibiotics were successfully discovered - this is called a ‘discovery void’- lack of new discoveries;

Efflux pump: Membrane proteins that function to export antibiotics out of the cell; Efflux pumps are considered an essential resistant mechanism;

Endothelial cells: Thin layer of cells, lining the interior surface of blood vessels and lymphatic vessels;

Epithelium: A type of animal tissue that lines the cavities and surfaces of blood vessels and organs throughout the body;

Fibroblasts: A type of cells responsible for making the extracellular matrix and collagen;

G proteins: Family of proteins involved in transmitting signals from different stimuli outside the cell to its inside; They can be regulated by the factors controlling binding and hydrolysis of GTP to GDP; The protein is active when GTP is bound and inactive when GDP is bound;

Glycoproteins: Proteins that contain oligosaccharide chains covalently bound to polypeptide side chains;

Gram positive bacteria: Bacteria with a cell wall consisting of a thick peptidoglycan layer, containing teichoic acids and lipoteichoic acids making the cell wall negatively charged; They retain their crystal violet dye after staining;

Gram negative bacteria: Bacteria that have a cell wall consisting of a thin peptidoglycan layer, and outer membrane; outer membrane consists of lipopolysaccharides, lipoproteins and phospholipids; they lose their dye after staining and become pink/red:

Hemorrhage: Technical term for bleeding;

Hemostasis: Process in which many cells of the immune system, are released to the injury site, creating a blood clot, which stops the bleeding at the wound site;

Homeostasis: The regulation of variables by a system in order to keep internal conditions stable;

Keratin: A protein, which protects the epithelial cells from damage;

Keratinocytes: Predominant cell type in the skin; act as a protective barrier against the external environment and warn the body to danger by e.g. producing cytokines;

Keratohyalin: A substance in granules located in the Stratum granulosom;

Lamellar granules: Secreted from keratinocytes, functions as a water-barrier for the skin;

Langerhans cells: Dendritic cells found in epidermis;

Lantibiotics: Class of peptide antibiotics, characterized by its thioether amino acid lanthionine or methyllanthionine;

Lipogenesis: A process in which acetyl-CoA is converted into a fatty acid;

Lipolysis: A process of breakdown of lipids; it involves hydrolysis of triglycerides into glycerol and free fatty acids;

Lipopolysaccharide: A molecule consisting of a lipid and a polysaccharide, forming the outer membrane of gram-negative walls;

Merkel cells: Epidermal cells localized in the Stratum Basale;

Melanocyte: Cells that produce the pigment melanin;

Negative cooperativity: Negative cooperativity is the decrease in binding affinity once one of the sites is bound;

Neutrophils: A group of highly phagocytic granulocytes; they are active in initial stages of infection; they have an ability to leave the blood, enter the infected tissue and destroy the microbes and foreign particles;

Psoriasis: A long-lasting autoimmune disease characterized by patches of abnormal skin, typically red, itchy and scaly;

Prophylaxis: A way to maintain health and prevent disease;

Proteoglycan: Glycosylated proteins found in connective tissue:

Ras protein: A family of proteins expressed in all cells and organs; involved in signal transmission within cells;

Re-epithelialization: Restoration of epithelium;

Stem cells: Cells that can differentiate into specialized cells or divide through mitosis to produce more stem cells;

TBE buffer: Tris/Borate/EDTA; buffer solution that contains Tris base, boric acid and EDTA; Tris-acid solution keeps the DNA water-soluble and deprotonated; EDTA is a substance, which binds specific ions, particularly Mg^{2+}, and remove them from the solution; these ions are needed co-factors for multiple enzymes; the EDTA's job is therefore to protect nucleic acids from enzymatic degradation;

Tonofilaments: A bundle of proteins and keratins, found in epithelial cells;

Appendix

1.0 Experimental procedure

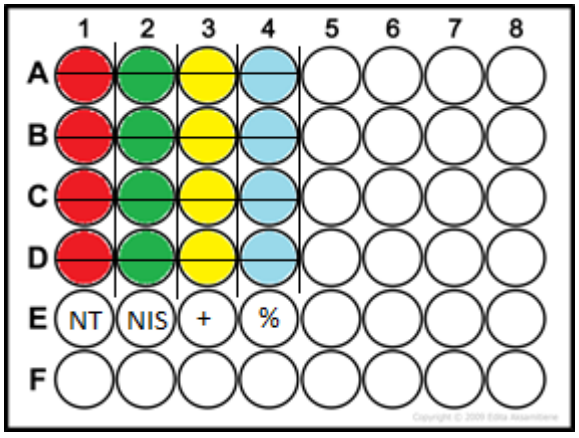
1.1 Subcultivating cells

The cells are grown on a flask surface in a 37°C, 5% CO_2 incubator.

a. Sterilize everything before placing it in the LAF bench
b. Remove media from flask
c. Wash cells twice with 10ml PBS
d. Remove PBS
e. Add 2mL trypsin
f. Incubate for 10 minutes
g. Add 8mL media (10% FBS)
h. Resuspend gently with a pipette 2-3 times
i. Count cells (Hemocytometer)
    i. Take out 10 µL cell culture
    ii. Count cells with click counter
j. Transfer 1 million cells to new flask (T75) and place in incubator

1.2 Scratch Assay

a. Check cells. If they are more than 70% confluent → scratch assay
b. Prepare a 48 well plate. The figure below is shown with neurotensin and nisin.



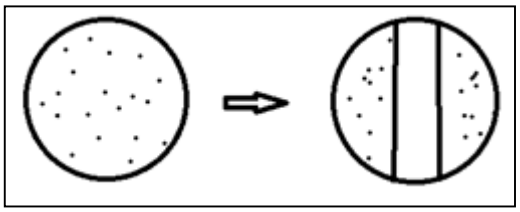
    i. In line E or F: Write what is added
        i. 1: A-D → 65.000 cells per well + 250 µL media
        ii. 2: A-D → 65.000 cells per well + 250 µL media

iii. 3: A-D → 65.000 cells per well + 250 µL media (no peptides)

iv. 4: A-D →250 µL media

ii. Horizontal lines are used as a starting point when measuring and taking pictures

c. Start scratch



d. Remove media from each well with a Pasteur pipette

e. Wash twice with PBS (250 µL)

f. Make 2x peptide stimuli master mixes

i. Neurotensin: media with 2% FBS

ii. Nisin: media with 2% FBS

iii. Media-FBS mix: 50 mL media (2% FBS) → 49 mL media + 1mL FBS

iv. Stock conc. Peptides: 2mg/mL (2000µg/mL). We want 25µg/µL

v. Wanted final volume: 1250 µL

vi. $C1 * V1 = C2 * V2 = \frac{1250\ \mu l * 25\ \mu g/ml}{2000\ \mu g/ml} = 15{,}6\ \mu L$

vii. 1250µL – 15.6µL = 1234.4 µL media to 15.6 µL peptide (Peptide stimuli master mix)

g. 250µL peptide stimuli is added to each well, neurotensin in one column and nisin in another

h. Measure scratch size and take pictures every 3 hours until the scratch heals

1.3 Cell harvest

1. Remove media with a Pasteur pipette

2. Wash cells twice with 250µL PBS (room temp.)

3. Harvest cells:

a. Add 100µL TRI reagent to the wells; resuspend gently with pipette x3

b. Transfer to Eppendorf tube (Well 1a → to tube 1a etc.)

c. Repeat and transfer to the Eppendorf same tubes (Well 1a → to tube 1a etc.) that already contain 100 µL

4. The total volume of the tubes will be 200µL

5. Place the tubes in -80 freezer

1.4 RNA isolation and measurements

When working with RNA, the samples have to be on ice and gloves must be worn at all times. As RNase degrades RNA, all articles used are zapped with RNase zap before put in the RNA fume hood. Everything is done in the RNA fume hood to prevent contamination.

1.4.1 RNA isolation and measurements procedure:

1. Turn the centrifuge on – set it to 4ºC and start it so it cools and will be ready for use
2. Get a styrofoam box and fill it up with ice
3. Get the 12 samples from the -80ºC freezer and put them on ice to defrost
4. Zap all articles and put it in the RNA fume hood
5. Calculate how much chloroform and isopropanol are needed for each sample
    a. 0.2mL chloroform for every mL of TRI reagent:

$$0.2mL * 0.2mL = 0.4mL = 40\mu L\ chloroform$$

    b. 0.5mL isopropanol for every mL of TRI reagent:

$$0.2mL * 0.5mL = 0.1mL = 100\mu L\ isopropanol$$

6. Dilute 96% ethanol with ddH_2O to get 75% ethanol

$$\frac{75\%}{96\%} = \frac{0.75}{0.96} = 0.78mL \rightarrow 0.78mL\ ethanol + 0.22mL\ H_2O = 1mL$$

$$4mL = 0.88mL\ H_2O + 3.12mL\ ethanol = 880\mu L\ H_2O\ \&\ 3120\mu L\ ethanol$$

1.4.2 Once the samples are defrosted and calculations are done:

7. Add 40µL chloroform to each tube, one tube at the time
8. Vortex for 15 seconds
9. Leave samples at room temperature for 10 min (phases starts to show)
10. Prepare 12 new tubes
11. Centrifuge at 4ºC, 12000g for 10 min (3 phases are now completely clear (clear, white, pink))
12. Transfer the clear phase from each centrifuged tube to each new tube
    a. Leave at room temperature for 10min
    b. Add 100µL isopropanol
    c. Vortex
13. Centrifuge at 4ºC, 12000g for 10 min

14. Make ethanol/H_2O mix (calculated amounts: 4 mL: 0.88 mL H_2O + 3.12 mL ethanol)
15. Discard supernatant (sup1)
16. Wash with 200µL ethanol/H_2O mix
17. Centrifuge at 4ºC, 7500g for 5 min
18. Discard supernatant (sup2)
19. Let samples air-dry for 10 min
20. Add 30µL Nuclease-free H_2O

1.4.3 Measurements:

21. Measure RNA concentration on nanodrop (260nm)
    a. Pipette 1 µL H_2O to zero the machine and use it as the blanc
    b. Measure 1 µL of each sample
22. Put samples back in the -80ºC freezer

1.5 cDNA

1.5.1 Prepare the master mix

1. Mix all master mix ingredients (+RT) in Eppendorf tube as on the table below:

Ingredients:	**Per reaction**	**9 reactions**
10x RT buffer	2.0 µL	18 µL
10x Random buffer primer	2.0 µL	18 µL
25x dNTP mix	0.8 µL	7.2 µL
Multiscribe™ reverse transcriptase	1.0 µL	9 µL
H_2O	4.2 µL	37.8 µL
Total volume	10.0 µL	90 µL

2. Resuspend gently with a pipette to mix the contents
3. Transfer 10 µL of the master mix to each labeled PCR tube
4. Mix all master mix ingredients (-RT) in Eppendorf tube as on the table below:

Ingredients:	**Per reaction**	**9 reactions**
10x RT buffer	2.0 µL	18 µL
10x Random buffer primer	2.0 µL	18 µL
25x dNTP mix	0.8 µL	7.2 µL
H_2O	5.2 µL	46.8 µL
Total volume	10.0 µL	90 µL

5. Repeat step 2 and 3
6. Add 10 µL RNA to all the tubes (in case of samples with high concentrations, 2µg is added and diluted with H_2O up to 10 µL)
7. Resuspend gently with a pipette to mix the contents
8. Spin down centrifuge for PCR tubes
9. Place the tubes in the rack on ice and transfer to Thermocycler

1.5.2 Thermocycler program:

1. Set the program in the Thermocycler according to the scheme below:
   - 95°C for 10 min
   - 37°C for 120 min
   - 85°C for 5 sec
   - 4°C for ∞
2. Set the tubes in the Thermocycler, make sure all tubes are properly closed
3. Start the program
4. When the program finish, transfer the tubes back to the lab and store in -18°C freezer until use.

1.6 PCR

1.6.1 Prepare the master mix

1. Mix all master mix ingredients in Eppendorf tube as on the table below:

Ingredients:	Per reaction	16 reactions
Hot start MM	7.5 µL	120 µL
Forward primer	1.0 µL	16 µL
Reverse primer	1.0 µL	16 µL
H_2O	3.5 µL	56 µL
Total volume	13.0 µL	208 µL

2. Resuspend gently with a pipette to mix the contents
3. Transfer 13 µL master mix to each labeled PCR tube
4. Add 2 µL cDNA to each tube
5. Resuspend gently with a pipette to mix the contents
6. Spin down in centrifuge for PCR tubes
7. Place the tubes in the rack on ice and transfer to Thermocycler

1.6.2 Thermocycler program

1. Set the program in the Thermocycler according to the scheme below:
   - 95°C for 10 min
   - 35 cycles:
     - 95°C for 30 sec
     - 52°C for 30 sec
     - 72°C for 30 sec
   - 72°C for 5 min
   - 4°C for ∞
2. Place the tubes in the Thermocycler, make sure all tubes are properly closed
3. Start the program
4. When the program finish, transfer the tubes back to the lab and store in -18°C freezer until use.

1.7 Gel electrophoresis

1.7.1 Pour the 1% agarose gel

1. Mix 1g agarose powder & 100mL TBE buffer
2. Microwave 2 min for the agarose powder to dissolve
3. Add 10 µL EtBr
4. Prepare gel tray; place well combs

5. Slowly pour the agarose into the tray to avoid bubbles
6. Let it sit for 30-60 min to solidify

1.7.2 Loading the samples

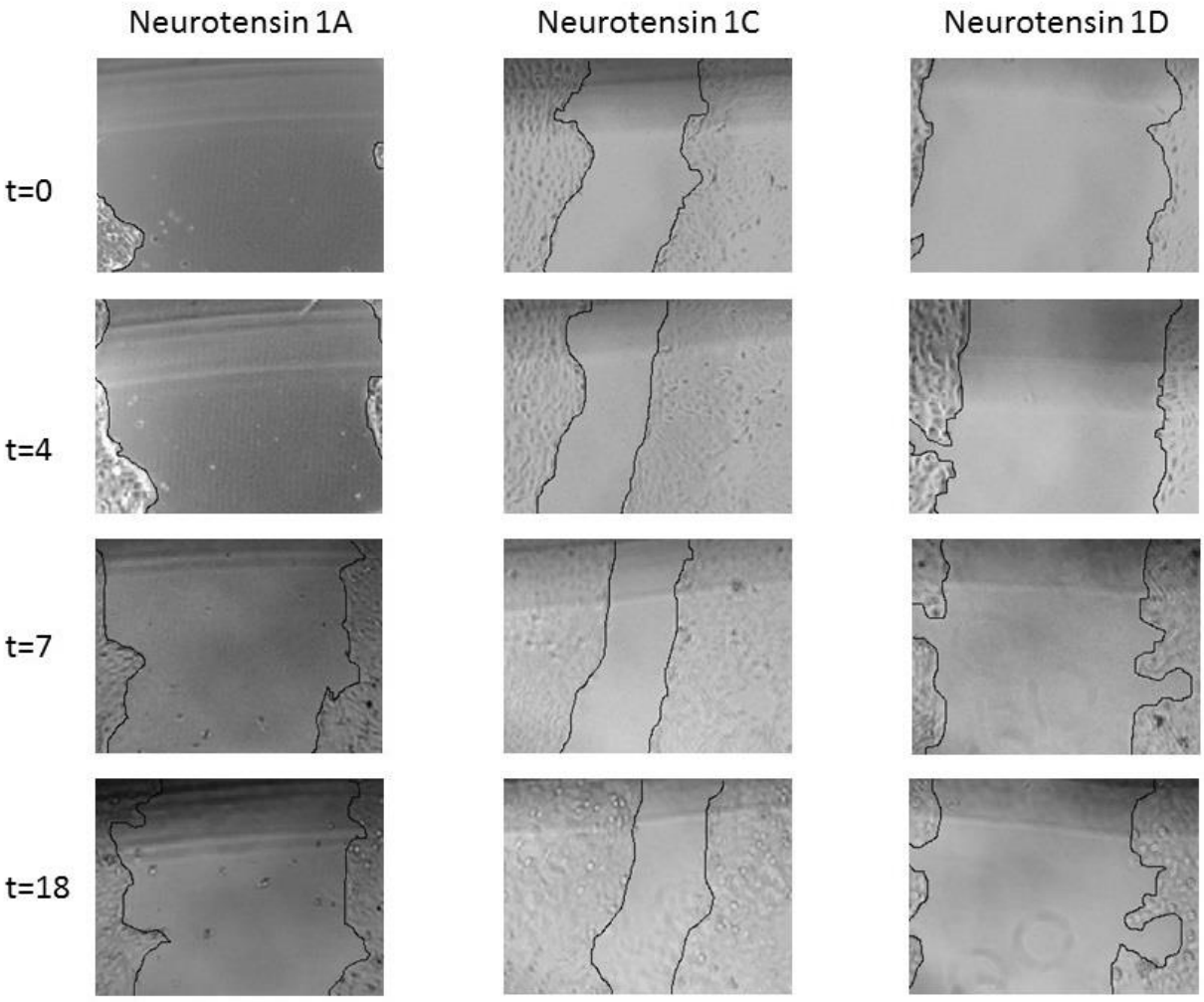
7. Place the agarose gel into the gel box
8. Add EtBr to the gel box/running buffer
9. Load 6µL ladder mixed with 4µL loading buffer into the first well
10. Add 4µL loading buffer to 7µL H_2O and load into the following well
11. Add 4µL loading buffer to 7µL of each PCR sample and resuspend
12. Load the samples into the following wells, and finish with another ladder, first with RT and then without: 1A, 1B, 1C, 1D, 2B, 3A, 1A-RT, 1B-RT, 1C-RT, 1D-RT, 2B-RT, 3A-RT.

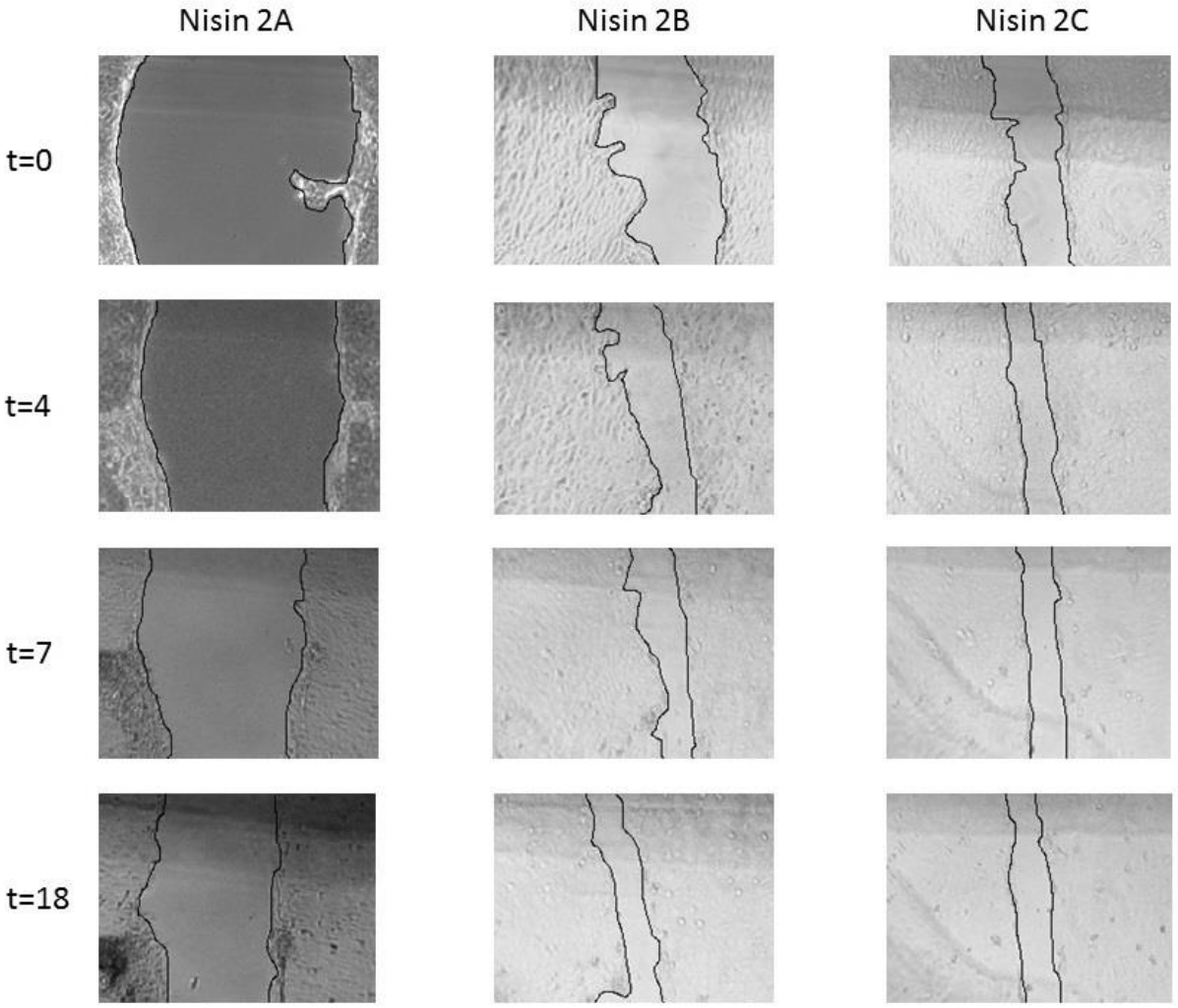
1.7.3 Run the samples & analyze the gel

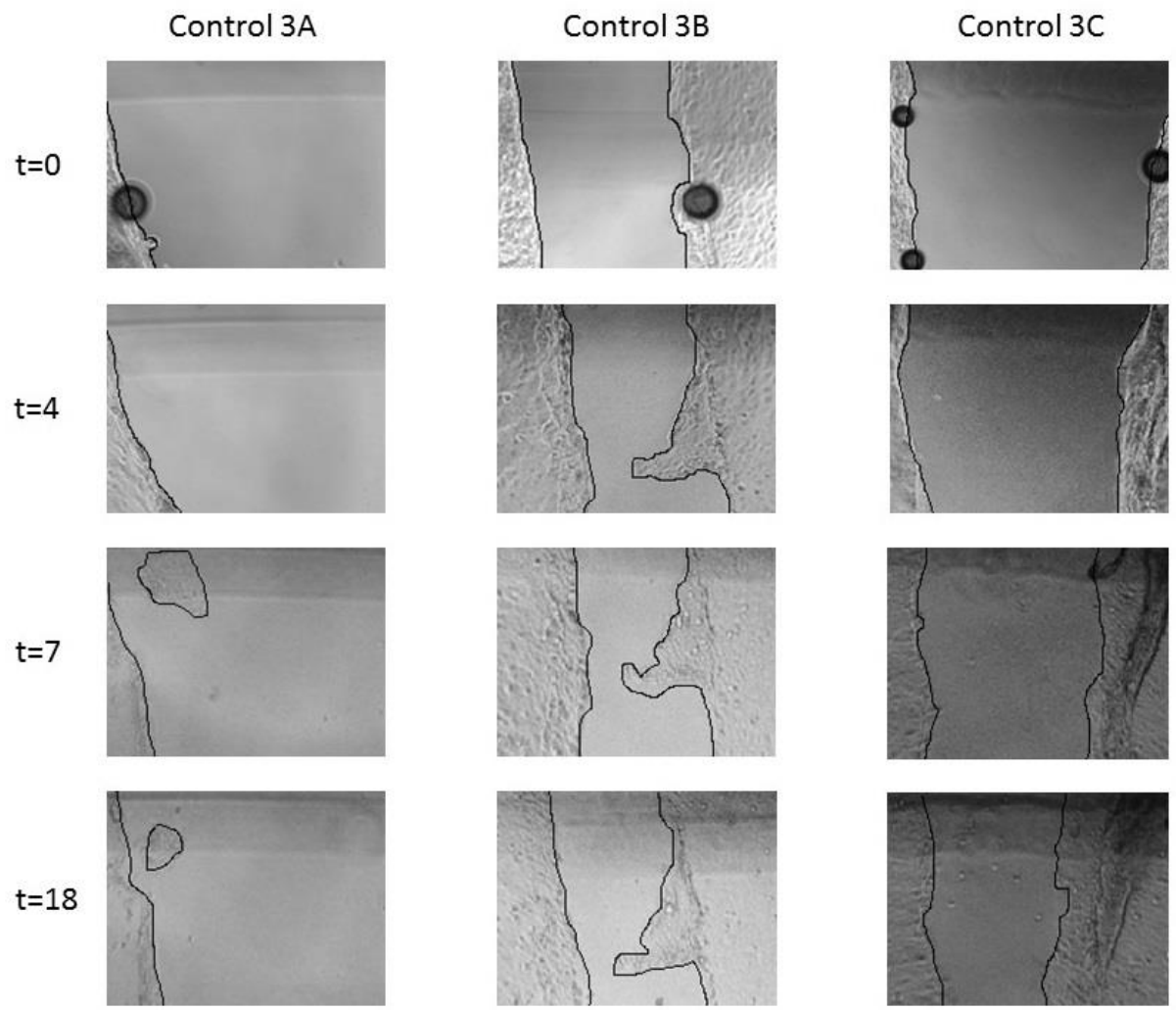
13. Run the gel at approximately 100V for 60-90 min
14. Electrodes: Black is negative, red is positive
    a. DNA is negatively charged → is pulled towards red electrode
15. Turn off the power
16. Remove the gel from the gel box
17. Use device with UV light to visualize the DNA fragments
18. Use the DNA ladder to interpret the bands' sizes

2.0 Results

Wound images




Nisin 2A
Nisin 2B
Nisin 2C
t=0
t=4
t=7
t=18



Average size of the wounds:

TID	**Average wound sizes**		
	Neurotensin	**Nisin**	**Control**
0	50	33.75	67.5
4	20.75	12.75	31.25
7	18.75	9.25	23.75
18	18.25	7.75	22

Healing in percentage:

	Neurotensin	**Nisin**	**Control**
A	64.71%	77.14%	68.00%
B	80.00%	86.67%	55.56%
C	60.00%	66.67%	76.47%
D	56.92%	70.00%	60.00%