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Maggot therapy's modes of action : effect of maggot secretions on microbiological, haematological and immunological processes

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General introduction and scope of this thesis

CHAPTER

ONE

From the time that we are young and obtain frequent cuts and bruises till later years when trauma, surgery, or illness may result in more extensive tissue damage, we repeatedly go through a series of events leading to repair of injured tissue. In most cases, wounds heal in a seemingly spontaneous manner. In some circumstances, however, the process of wound healing is interrupted and scar formation is delayed. Wound healing is a complex process that can be roughly divided into three overlapping phases: inflammation, proliferation and remodelling (Figure 1). To ensure a positive outcome, wound healing processes are strictly regulated by cell-cell contact and the action of multiple cytokines, chemokines and growth factors released at the site of injury. Unfortunately, wound healing is fragile and subject to failure, and may lead to the formation of chronic wounds. Such wounds may benefit from maggot therapy, i.e. the application of the larvae of certain flies to the open wound. In this thesis, the effects of maggot excretions/secretions on various processes of wound healing are investigated.

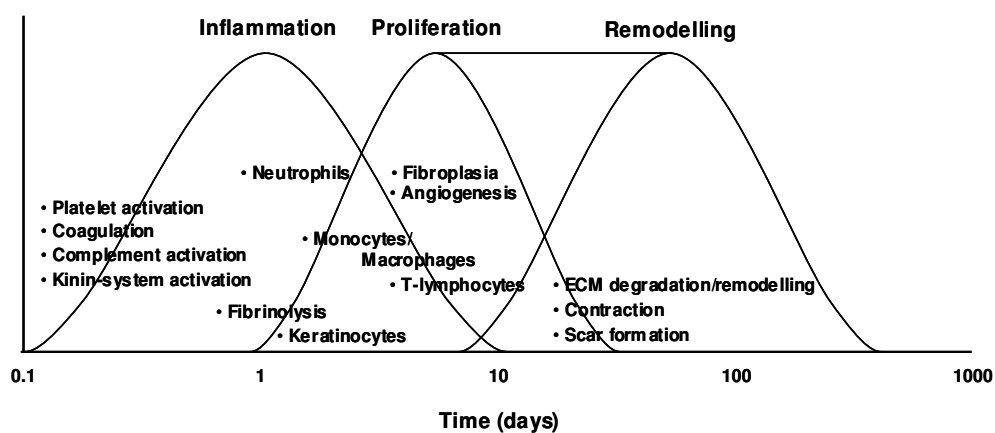


Figure 1 The three phases of wound healing (adapted from S. Enoch and P. Price. Cellular, molecular and biochemical differences in the pathophysiology of healing between acute, chronic and aged wounds. *World Wide Wounds*, August 2004)

The inflammatory phase

Four major plasma enzyme systems play a role in the control of inflammation: the clotting, kallikrein-kinin, complement and fibrinolytic systems. Following damage to capillary blood vessels an immediate reflex promotes vasoconstriction which slows the blood flow. This enhances platelet adhesion and activation through exposure to thrombogenic components, such as collagen, at the damaged site and leads to the formation of a platelet clot^{1,2}. The damaged tissue and activated platelets then produce factors that activate a coagulation signalling cascade leading to the formation of a fibrin clot. The clot contains plasma-derived

glycoproteins, such as fibronectin and vitronectin³, and plasminogen amongst others, and traps platelets, leucocytes and red blood cells⁴. The blood clot serves as a provisional extracellular matrix allowing cells to migrate into the injured area; fibrin and fibronectin are the most abundant proteins in the provisional matrix. The activated kallikrein-kinin system triggers the release of vasoactive kinins which are involved in vasodilatation and increased vascular permeability⁵. This process resembles that of histamine released from mast cells. The complement system comprises three distinct pathways leading to the formation of factors involved in opsonisation of micro-organisms for ingestion by phagocytes, lysis of micro-organisms, chemotactic attraction of phagocytes, processing of immune complexes and the activation of immune cells⁶. Finally, the fibrinolytic system is responsible for the degradation of fibrin clots and plays a role throughout the different phases of the wound healing process. Hallmarks of fibrinolysis are the formation of plasmin from fibrin-attached plasminogen by plasminogen activators and the subsequent degradation of fibrin by this enzyme².

The cellular response to tissue damage starts with the activation of resident cells. Within 24 h of injury, neutrophils are the first to arrive at the injury site in response to chemotactic factors derived from activated platelets, resident (dying) cells and infectious micro-organisms as well as fibrin degradation products and complement factors (C5a and C3a). Efficient recruitment of cells from the circulation into the site involves tethering, rolling and firm adhesion to the endothelial cell surface and finally diapedesis. Regulation of these initial steps involves selectins and integrins that recognize cell surface receptors or matrix proteins such as fibrin, fibronectin and vitronectin^{3,7}, whereas different molecules are involved in diapedesis (e.g. PECAM and CD99)⁸. Neutrophils are predominant in the first 2-3 days after injury and the numbers peak at around 48 h. Their main function is to eliminate dead cells and micro-organisms by phagocytosis and subsequent destruction in the phagolysosome using oxygen-dependent and -independent mechanisms. The oxygen-dependent mechanisms involve NADPH oxidases which use molecular oxygen to produce superoxide anions (O_2^-) and which can be converted to hydrogen peroxide (H_2O_2)⁹. The enzyme myeloperoxidase, present in azurophil granules, converts H_2O_2 to hypochlorous acid. The oxygen-independent mechanisms involve degranulation of the granule subsets (azurophil, specific and gelatinase granules and secretory vesicles) into the phagolysosome¹⁰ as well as the extracellular micro-environment. Taken together, the actions of reactive oxygen species and granule contents, including enzymes, antimicrobial peptides and proteins, lead to the destruction of ingested as well as extracellular micro-organisms.

Within 48 h after injury, monocytes are initially attracted to the wound by some of the same chemoattractants that trigger neutrophils. Whereas neutrophil numbers decline after a couple of days, the recruitment of monocytes continues through monocyte-specific chemoattractants¹¹. In response to local factors at the wound, monocytes may differentiate to macrophages which then become the predominant cell type later in the inflammatory

phase (around day 5). In addition to clearing the wound of bacteria and tissue debris, monocytes and macrophages in particular regulate the inflammatory process by secreting cytokines, chemokines and growth factors. Initially, monocytes differentiate mainly into pro-inflammatory macrophages. These cells display high levels of pro-inflammatory cytokines and chemokines, which are responsible for the recruitment and activation of additional leucocytes¹² and Th1 lymphocytes. Activation of Th1 cells is further induced by the expression of co-stimulatory molecules on the macrophages and by antigen processing and presentation^{13,14}. Th1 lymphocytes activate pro-inflammatory macrophages, thus further enhancing the pro-inflammatory responses. At the end of the inflammatory phase, when most of the infectious agents and tissue debris are cleared, the balance shifts from pro-inflammatory macrophages to macrophages with anti-inflammatory/pro-angiogenic cytokine and growth factor activities. These cells suppress inflammatory responses both directly¹⁵⁻¹⁷ and indirectly by inducing regulatory T cells¹⁸. They also mediate the clearance of apoptotic cells^{16,19} and induce neovascularisation and fibroblast and epidermal cell proliferation²⁰, thereby playing a pivotal role in the transition from inflammation to repair.

The proliferation phase

The main characteristic of the proliferation phase is the replacement of the provisional matrix with newly formed granulation tissue. This process lasts for about two weeks after wounding.

Granulation tissue formation, the process that ensures reconstitution of the dermis, starts within 4 days after injury. In response to growth factors derived from macrophages and keratinocytes, fibroblasts at the wound edges proliferate and migrate into the provisional matrix, which they then degrade by activation of the fibrinolytic system. In the meantime, secretion of basement membrane components, such as collagen, glycosaminoglycans and glycoproteins such as fibronectin and tenascin²¹⁻²³, results in the synthesis of a new collagen-rich matrix, a process termed fibroplasia. Fibroblasts that have migrated to the wound site produce growth factors to further facilitate protein and extracellular matrix (ECM) synthesis. The main function of fibroblasts is the production of new ECM which serves as a scaffold for collagen fibrils and facilitates migration of keratinocytes, fibroblasts and endothelial cells. Binding of these cells to the ECM is mainly facilitated by integrin receptors. The ECM serves also as a reservoir and modulator for (inactive) growth factors²⁴ and mediates wound contraction^{25,26}.

To provide nutrients and oxygen to the newly formed granulation tissue, new capillaries/blood vessels are formed by sprouting of pre-existing ones. This process, termed angiogenesis, consists of the activation of endothelial cells by macrophages, degradation of their basement membrane, outgrowth into the wound/new tissue, cell proliferation and migration into the perivascular space, tubule formation, basement membrane reconstitution, formation of new capillary loops and finally re-establishment of the blood flow²⁷. There are

several factors that stimulate angiogenesis including growth factors, hypoxic and high-lactate wound environment and low concentrations of reactive oxygen species²⁸. Clearly, granulation tissue formation and angiogenesis are overlapping processes. New vessels are essential to support the forming matrix, which in turn supports the new capillary network

Re-epithelialisation is the process of restoring the epidermis and is induced by the presence of several growth factors produced by macrophages, fibroblasts and keratinocytes. Within 24 h after injury keratinocytes start migrating from the wound edges using surface integrin receptors to interact with the provisional matrix while separating eschar and debris that may cover the wound from the newly developing granulation tissue²². This process, which involves the degradation of the matrix, is part of the fibrinolytic system. In addition to cleaving plasminogen to form plasmin, plasminogen activators also activate collagenases, which together facilitate the degradation of the ECM and fibrin eschar in the direction of the migration of the cells¹. To ensure sufficient cell numbers for coverage of the wound, proliferation of keratinocytes located close to the migrating cells is increased while the proliferation potential of migrating keratinocytes is inhibited²⁹. When migration ceases, due to contact inhibition, keratinocytes attach to the underlying substratum and differentiate to generate a stratified epidermis.

The remodelling phase

Remodelling occurs throughout the entire wound healing process. The provisional matrix is replaced by granulation tissue which contains type III collagen and newly formed blood vessels, and subsequently is replaced by a collagenous scar predominantly containing type I collagen with less mature blood vessels²⁶.

Wound contraction is the process that leads to the reduction of the wound area. The degree of contraction depends on the depth of the wound. During granulation tissue formation, fibroblasts undergo phenotypic modulation and differentiate to myofibroblasts, which are characterised by the presence of α -smooth muscle actin fibrils along the plasma membrane. The classical view is that these cells are primarily responsible for contraction by extension of pseudopodia. These facilitate the binding of cytoplasmic actin to extracellular fibronectin and collagen fibres, leading to retraction which draws the collagen fibres to the cell^{25,30,31}. However, two contrasting studies reported myofibroblasts are not required for contraction^{32,33}. Instead, fibroblasts were shown to influence contraction by reorganizing collagen fibrils rather than pull on the surrounding tissue. Therefore, the mechanisms of wound contraction are unclear and need further exploration.

Approximately 80% (dry weight) of the normal dermis consists of collagen fibres which provide structure, strength and rigidity to the tissue³⁴. Within the first week after injury, fibroblasts produce type III collagen to form granulation tissue. However, this collagen is unstructured and does not provide the necessary strength. Therefore, the collagen fibres have to be remodelled and this occurs by degradation of type III collagen and subsequent

synthesis of type I collagen. The degradation of type III collagen depends on the presence of matrix metalloproteinases (MMPs) and their inhibitors produced by macrophages, keratinocytes and fibroblasts in response to cytokines, growth factors and/or cell contact with the ECM³⁵. In addition, the newly formed vasculature undergoes remodelling by regression and involution leading to fewer mature vessels²⁷. Remodelling continues for up to 2 years but the resulting scar contains fewer cells than normal skin and only reaches up to 70% of its pre-injury strength.

Impaired wound healing

Wound healing is a well-orchestrated but fragile process and is subject to failure to progress through one of its phases (Figure 2), leading to the development of chronic, non-healing wounds. This may result in decreased physical, emotional and social function of patients and therefore a reduced quality of life. In addition, such wounds, which are one of the most common causes of non-traumatic amputation, result in major economic costs for the patients, their families and Society as a whole³⁶. Chronic wounds mostly affect people over the age of 60 and the incidence of these wounds is expected to increase.

Impaired healing of wounds can be induced by numerous factors both local and systemic. Local factors include the presence of foreign particles or micro-organisms, ischaemia, tissue maceration, callus formation, pressure and infection whereas systemic factors comprise malnutrition, age, vascular insufficiencies, immune suppressive medication and underlying conditions such as diabetes mellitus. The majority of chronic wounds occur at the lower extremities and can be classified into three categories: venous ulcers, diabetic ulcers and pressure ulcers.

Bacterial infection

Colonisation and infection of the wound surface by bacteria contribute to the failure of wound healing³⁷⁻³⁹. High levels of several bacteria can induce lysis of clots and/or the extracellular matrix^{40,41}. This results in impaired cell migration and/or proliferation of leucocytes and fibroblasts, which leads to a delayed immune response. Consequently, bacteria can spread more easily thereby establishing an infection. When leucocytes arrive at the affected site, they initiate a substantial pro-inflammatory response to fight the bacterial infections. However, the bacteria may have formed biofilms, as is often observed in chronic wounds⁴². Due to altered growth characteristics and gene expression profiles⁴³, these bacteria are protected against the actions of antibiotics^{44,45} and cells and effector molecules of the immune system^{39,46}. Stimulation of pro-inflammatory responses therefore continues unabated. In general, bacterial levels above 10^5 organisms/gram of tissue are associated

with poor healing²⁸. Clearly, open wounds, especially the presence of necrotic tissue, offer an opportunity for bacterial entry and proliferation.

A large variety of bacterial species have been identified in chronic wounds. Commonly found organisms include gram-positive *Staphylococcus aureus*, *Enterococcus* spp, and *Streptococcus* spp, and gram-negative species such as *Pseudomonas aeruginosa*, *Enterobacter* spp and *Serratia* spp. Furthermore, anaerobic bacteria have also been reported including *Peptoniphilus* spp, *Fingoldia magna* and *Clostridium* spp^{47,48}.

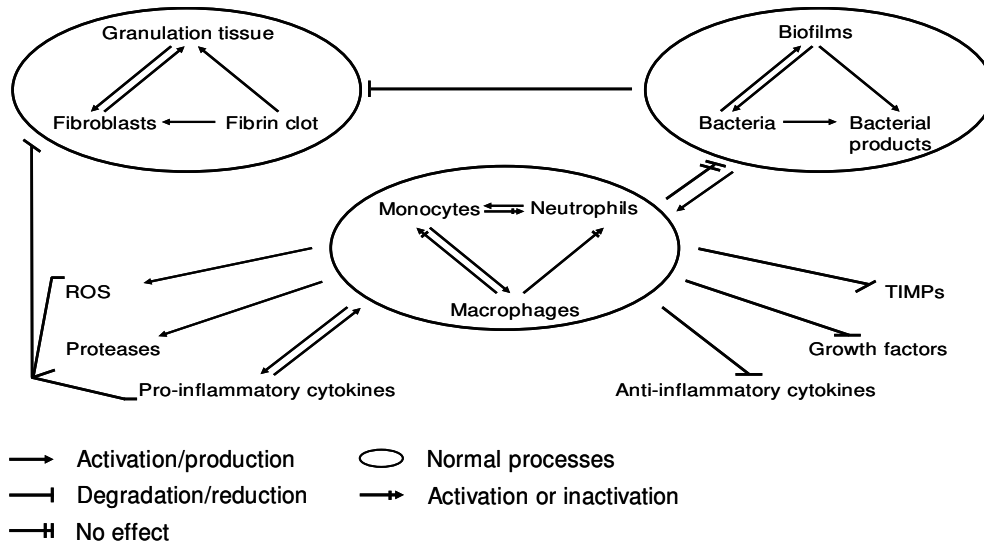


Figure 2 Processes in chronic wounds
 ROS, reactive oxygen species; TIMPs, tissue inhibitor of metalloproteinases.

Enhanced inflammatory responses

Although pro-inflammatory responses are essential for wound healing, they become detrimental in wounds where inflammation persists. In the cases where bacteria cannot be eliminated, leucocytes in the wound continue to produce pro-inflammatory mediators. Consequently, the influx of new leucocytes, such as neutrophils, monocytes and macrophages, increases⁴⁹⁻⁵¹. This leads to excessive pro-inflammatory responses in these wounds, which attract even more cells that also produce pro-inflammatory cytokines^{52,53}. Phagocytes are activated to release proteolytic enzymes and also to produce large amounts of reactive oxygen species (ROS)^{54,55} as a consequence of pro-inflammatory cytokines and/or bacterial products present in the wound. In agreement with this, chronic leg ulcers are associated with elevated expression of pro-inflammatory cytokines, such as TNF- α ,

compared to normal healing wounds⁵⁶⁻⁵⁸; the levels of these cytokines decrease when the wound begins to heal. Moreover, neutrophils of patients with chronic conditions, such as chronic venous insufficiency⁵⁹ and posttraumatic osteomyelitis⁶⁰, are primed to produce high amounts of superoxide anion upon exposure to stimuli.

Enhanced proteinase activity

Increased levels of pro-inflammatory cytokines enhance the synthesis and/or release of several matrix metalloproteinases and serine proteases^{3,53,61}, whereas ROS augment the effects of these proteinases^{62,63}. In agreement with this, increased proteolysis has been observed in chronic wounds. Elevated levels of MMP-1, MMP-2, MMP-8 and MMP-9 have been reported for diabetic⁶⁴, pressure⁶⁵ and venous ulcers^{66,67}, as compared to normal healing wounds. Altered distribution of proteinase-producing cells in specific wound areas has been observed⁶⁸. In addition, levels of TIMPs are found to be decreased in chronic wounds^{64,66}. A possible explanation for this could be that excess levels of proteinases^{3,69-71} and ROS^{62,63} cause proteinase inhibitor inactivation. Taken together, in chronic wounds the balance between MMPs and TIMPs appears to be disturbed favouring wound degradation. Of note, some contrasting reports have been published⁷².

Other proteinases, such as elastase released from azurophil granules, have been reported to be elevated as well in chronic wounds^{59,73} due to the large numbers of activated neutrophils. Interestingly, one study showed that elastase degrades MMPs *in vivo* and the authors suggested that elastase is the main cause of ECM destruction⁷⁴.

Impaired matrix synthesis and composition

Excess proteinase activities cause destruction of the matrix (and newly formed granulation tissue) by degradation of its components, such as fibronectin, vitronectin and tenascin-C^{3,64,75,76}. This leads to impaired cell migration and/or proliferation of fibroblasts, keratinocytes and endothelial cells. Consequently, the mechanical obstruction of re-epithelialisation, wound contraction and remodelling may enhance bacterial infection and prolong the inflammatory response. Moreover, this may lead to the development of fibrin slough and necrotic tissue.

Ulcers may be caused by decreased fibrinolytic activity. In chronic conditions such as obesity and diabetes, levels of plasminogen activator inhibitor 1 (PAI-1) are enhanced^{77,78}, probably due to elevated levels of inflammatory mediators such as TNF- α and C5a^{79,80}. PAI-1 binds to and inactivates plasminogen activators, resulting in impaired lysis of pericapillary fibrin cuffs and subsequent causes ulcer formation^{77,81}. In addition, enhanced levels of methylglyoxal are found in diabetic patients resulting in decreased activation of plasminogen activators⁸², thereby decreasing fibrinolysis even more. In addition to inducing ulcer formation, decreased fibrinolysis of the provisional matrix has been associated with delayed re-epithelialisation and reduced migration of fibroblasts and keratinocytes⁸³⁻⁸⁶.

Altered growth factor production and/or signalling

Chronic wounds may differ in the levels of growth factors and/or in the cellular responses to these factors from normal healing wounds. It has been reported that growth factors such as PDGF, TGF- β , IGF, FGF and VEGF, which are involved in the recruitment and stimulation of cells that are responsible for repair, are decreased in chronic wounds⁸⁷⁻⁹⁰. However, other reports mention no local growth factor deficiency in chronic wounds^{57,91,92}, whereas increased levels of PDGF and VEGF have been found as well^{93,94}. Of note, increased levels of VEGF in chronic wounds were accompanied by increased levels of the VEGF inhibitor^{95,96} and/or its degradation⁷⁰. These contrasting results for the levels of growth factors may be caused by differences in wound pathology and cell types or localisation of the cells within the wound and should be further investigated. Furthermore, the mechanisms underlying the imbalances in growth factors and their inhibitors in chronic wounds remain to be elucidated. Excess levels of proteinases^{69-71,97} and ROS^{62,63} may cause growth factor degradation/inactivation. Furthermore, growth factors may bind to protein macromolecules and become 'trapped' so that they are unable to bind and activate cells²⁸. Another possible explanation is that an impaired ECM composition diminishes the actions of growth factors *via* a decrease in integrin binding³. Finally, intracellular signal transduction may be dysfunctional in the cells⁹⁸.

Changes in cellular profile and activity

Cellular profiles and activities in chronic wounds differ from those in normal healing wounds. Leucocytes produce excessive pro-inflammatory mediators, as indicated above. Pro-inflammatory cytokines have been shown to inhibit proliferation and induce morphological changes in normal skin fibroblasts⁹⁹. These cells become senescent (the process of growing old) as a consequence and have a diminished or even lost the ability to respond to growth factors^{100,101}. Additionally, these cells may also be in a state of cell cycle arrest and therefore unresponsive to signalling proteins¹⁰². In agreement with this, fibroblasts derived from chronic ulcers display a decreased proliferative response to growth factors due to impaired intracellular signalling^{98,103,104}.

Angiogenesis is impaired in chronic wounds despite enhanced levels of VEGF and, as indicated above, enhanced levels of VEGF inhibitor or degradation of VEGF could be responsible. Another possible explanation is that excess levels of MMPs degrade the ECM thereby impairing endothelial cell migration. In agreement with this, a MMP inhibitor partly restored tubule formation in the presence of chronic wound exudate¹⁰⁵. However, the effects of factors present in chronic wounds on endothelial cells are unknown.

Finally, epithelialisation is often impaired in chronic wounds, due to impaired migration of keratinocytes. A possible explanation could be the protease-induced degradation of the ECM. Furthermore, impaired signalling, decreased expression of surface receptors

necessary for (growth factor) binding and migration, or increased apoptosis have been proposed³.

Treatment modalities

Many treatments are available that may induce healing of chronic wounds. These treatments may involve different aspects of wound bed preparation including a restoration of the bacterial balance (e.g. antibacterial agents and dressings), the management of necrosis (debridement), the management of exudate (e.g. dressings, high compression bandaging and vacuum-assisted closure), and the correction of cellular dysfunction and biochemical balances (e.g. growth factors [PDGF-BB], ECM components and bioengineered skin containing fibroblast and/or keratinocytes)¹⁰⁶. Debridement refers to the removal of damaged, infected and/or dead tissue from the wound bed. Removal of necrotic tissue makes it easier to obtain a moist environment and leads to a better assessment of the wound or ulcer. Furthermore, many bacteria are removed simultaneously, which reduces the bacterial load in the wound. Additionally, debridement removes senescent cells. Besides the clinical relevance, debridement reduces psychological stress due to the bad odour and the appearance of the wound, and it leads to an improved clinical and cosmetic outcome. There are several ways to debride a wound including surgical, mechanical, chemical, enzymatic and autolytic methods. In this thesis maggot debridement therapy is considered in detail.

A history of maggot therapy

Although it has been reported¹⁰⁷ that maggot therapy was used by several peoples such as the aboriginals in Australia, the hill peoples of Northern Burma and possibly the Maya in Central America, the beneficial effects of myiasis (maggot infestation) are not universally recognised or appreciated.

Most knowledge about the treatment of wounds in 16th Century Europe is obtained from a book written by Ambroise Paré (1509-1590), chief surgeon to Charles IX and Henri III. In his first 'Journey' he describes a passage from the book 'Of wounds in General' eighth chapter written by John de Vigo stating a frequently applied method to cure wounds made by firearms: "...to cauterize them with oyl of Elders scalding hot, in which should be mingled a little treacle..."¹⁰⁸. In the same period, a surgeon from Turin, who was famous for his treatment of gunshot wounds, used a balm made of new born whelps boiled in the oil of lilies and prepared earthworms with Venetian turpentine¹⁰⁸. Paré is the first Western surgeon who described human myiasis on several occasions. He stated, in reference to a patient with a bad skull wound: "*Now to take away this corruption, I applied at certain times actual cauteries...: but mark, after some months space, a great number of worms came forth by the holes of the rotten bones from underneath the putrified skull...The bone which nature separated was of the bigness of the palm of ones hand... and yet the patient not dye thereof; for he recovered yet beyond all means of expectation*"¹⁰⁹. Due to this sentence, Paré is regarded by many as one of the first surgeons to recognize the beneficial effects of maggots in the healing of wounds. However, the following two statements make clear that this is not the case: "*what marvail was it, if in these late civil wars, the wounds which were for their quantity small... have caused so many and grievous accidents... Especially, feeling that the Air which encompasseth us, tainted with putrefaction, corrupts and defiles the wounds by inspiration and exspiration... And the corruption was such, that if any changed to be undrest for one day, ... the next day the wound would be full of worms*"¹¹⁰. The second statement from Paré, when describing the battle of St. Quintin in 1557 is: "*The wounds of the hurt people were greatly stinking and full of worms with gangrene and putrefaction; so that I was constrained to come with my knife to amputate that which was spoiled. Now their were not any medicines... neither was there half enough to dress so great a number of the people, ... and to kill the worms that were entred into their wounds...*"¹¹¹.

The first known description of beneficial effects of myiasis was recorded by Baron D.J. Larrey (1766-1842), inspector-general of the medical department of Napoleon's army. He wrote: "*The presence of these maggots in the wounds appears to hasten the completion of the suppuration; it caused also an inconvenient itching to the patient, and obliged us to dress the wounds three or four times in the day*"¹¹². Further observations of favourable wound myiasis, made during the American civil war, came from the confederate surgeons Joseph Jones¹¹³, who investigated the causes of disease and death in confederate prisons

and hospitals, and John F. Zacharias, who is regarded as the first Western physician to intentionally introduce maggots into wounds for debridement (as described in his obituary)¹¹⁴.

The founder of modern maggot therapy is the orthopaedic surgeon William Baer (1872-1931). During the First World War, Baer treated two soldiers who had lain on the battlefield for seven days. Although having serious injuries, "...they had no fever and there was no evidence of septicaemia or blood poisoning. ... On removing the clothing from the wounded part, much was my surprise to see the wound filled with thousands and thousands of maggots, apparently those of the blow fly. ...these wounds were filled with the most beautiful pink granulation tissue that one could imagine"¹¹⁵. In 1928, Baer put his observations into practice by successfully treating his first patients with maggots and, after having some problems with maggot-induced infections, developed a method to sterilize and cultivate the larvae. From 1930 on, maggot therapy became a popular and widespread method for the treatment of infected wounds leading to over 53 publications within the first 5 years¹¹⁶. Several reports were published on cheaper ways of capturing, rearing and sterilising maggots as well as on comparing different ways of applying the maggots¹¹⁷⁻¹²⁰, as Baer's method of rearing and sterilising maggots was relatively expensive. In addition, reports were published comparing maggot therapy to several other treatments¹²¹. Furthermore, the bactericidal activities of maggots^{122,123} and the wound healing properties^{121,124-126} were investigated thoroughly. Although observations by different researchers led to contrasting conclusions^{122,123,126-128}, it was generally agreed on that the beneficial effects of maggots were not solely caused by mechanical removal of necrotic tissue but that other factors were involved as well.

In 1935, the results were published of a questionnaire which was sent out to 947 surgeons known to have used maggot treatment both in the USA and Canada¹¹⁶. 605 surgeons returned the form leading to a total of 5750 cases; 91.2% of the users expressed a favourable opinion (95.3% of the cases) while the remaining 8.8% was neutral or critical. The major objections of the latter group were the costs of obtaining the maggots, pain and discomfort of patients, along with the time and trouble in applying the treatment. Most research on the use of maggot therapy during this time period was probably published by S.K. Livingston. In 1936, he published a report on the clinical application of maggots and/or maggot 'active principle' in 567 patients¹²¹. Granulation tissue formation was observed in 88% of the cases leading to hospital discharge. This success rate was 38% higher as compared to other treatments. In addition to maggot treatment, a vaccine therapy was administered intramuscularly consisting of pyogenic organisms suspended in the 'active principle' as a vehicle. However, no results were mentioned and no further references were made about this vaccine therapy possibly due to unfavourable systemic reactions. In 1937, Livingston published another report on the use of 'active principle' of maggots; of the 1020 cases, 415 were treated with living maggots in combination with the active principle while

605 cases were treated by maggot extract alone¹²⁹. The results showed a 60 to 100% clinical improvement, depending on the type of wound, although the extent of improvement differed between the wound types. A year later, Livingston published a preliminary report on the use of a grease-free jelly containing 5% of the 'active principle'; although he reported beneficial effects on healing, no information was given on the consistence of the jelly¹²⁷.

At the end of the 1930s, the development of improved surgical techniques and the discovery and distribution of antibiotics made maggot therapy obsolete^{107,128,130}. In the following 50 years, maggot therapy was used only as a last resort and became largely forgotten¹³¹. In 1986, E. Chernin wrote a short review on maggot therapy as he found the 'story of the maggots brief and largely forgotten moment on the surgical stage' worth retelling: "*However unlikely they may seem now as agents of human health, the lowly maggots worked diligently and well. We have since then restored them to their accustomed place as vermin*"¹³². Amusingly, Chernin refers to an article published in 1983 by Pechter and Sherman as "*maggot therapy came and went within a decade or so, though some suggest that the technique may one day reappear*".

Research into maggot therapy

In the 1930s a large number of experiments were carried out to optimise maggot therapy and also to isolate active components from maggots and their excretions/secretions. Since its re-introduction in the late 1980s and early 1990s, the number of publications dedicated to this therapy has been rising.

Type of flies

Many species of the Diptera family Calliphoridae (blowfly) are capable of infesting living hosts (myiasis). These myiasis-causing flies can be grouped into two categories: obligate and facultative parasites. Obligate parasites can cause severe damage to healthy tissue as these larvae need living tissue and are therefore unsuitable for maggot therapy. Facultative parasites can feed on living tissue but more commonly use dead/necrotic tissue as their source of nutrition.

Already in the 1930s, practitioners understood the importance of selecting the most suitable fly for maggot therapy. Baer reported the satisfactory use of the blue-black bottle fly *Phormia regina*, the green bottle fly *Sucilia (Lucilia) sericata* and *Lucilia caesar*¹¹⁵. In addition, Weil *et al* reported that the large blue bottle flies (*Calliphora vomitans* and *C. erythrocephala*) could be used as well¹¹⁷, whereas Fine and Alexander were more in favour of *Lucilia cuprina*¹¹⁹. The most common type of fly used for therapy was *Lucilia sericata* probably because this species was shown to starve on clean granulation tissue¹¹⁷. Choosing the right type of fly was not always easy, as noted by Buchman and Blair^{117,133}. One of their

batches of larvae bored large cavities in the healthy granulation tissue thereby increasing the size of the wound; instead of using *L. sericata* larvae they probably used the similar looking Texas screw-worm larvae which are obligate parasites¹¹⁹.

The first commercial supplier of maggots was Lederle (1932), which sold 1000 maggots for five dollars^{117,118}. This led to an average treatment cost of 55 dollars per patient. Practitioners searched for ways to rear the larvae cheaper themselves as this was too expensive for many hospitals. For example, Fine and Alexander reported that they obtained their original laying stock by exposing fresh meat in the open near a meat market^{117,119}. Nowadays, the maggots of *Lucilia sericata* are used most frequently and are easily available from commercial suppliers such as BioMonde in Germany, Zoobiotic in the United Kingdom and MonarchLabs in the United States. However, hospitals in many countries still rear their own maggots due to costs or transportation problems.

Debridement

The effects of maggots on wounds can be divided into three general mechanisms: debridement, antibacterial effects and stimulation of wound healing.

Debridement is the most known and widely accepted mechanism of action by maggots. This is emphasized by the FDA's 2004 approval of maggots as a medical device to clean out wounds (hence the name maggot debridement therapy). Maggots likely debride wounds by secreting proteolytic enzymes/peptides which dissolve the necrotic tissue. Numerous enzymes have been reported including collagenases^{134,135} and serine proteases (trypsin-like and chymotrypsin-like)^{136,137}, carboxypeptidases A and B, leucine aminopeptidase¹³⁸, lipases¹³⁹, a metalloproteinase and an aspartyl proteinase¹³⁶. Subsequently, maggots ingest the liquefied tissue which may contain bacteria, cellular debris, and serous drainage of the wound. In addition, the mechanical action of wriggling maggots might enhance debridement, as the maggots probe and macerate the necrotic tissue with their mouth hooks. Together, the secretion of proteolytic enzymes, the ingestion of the resulting liquefied tissue and possibly the mechanical action of maggots result in an efficient debridement of necrotic wounds.

Antibacterial effects

Debridement by maggots results in a wound environment that is less susceptible to bacterial colonisation. Furthermore, as maggots ingest the dissolved tissue, they take up large numbers of bacteria. In 1933, Robinson and Norwood observed that ingested bacteria were abundant in the fore stomach of maggots while the hind stomach showed only slight growth in one-third of the cases¹²². The intestines of the maggots were sterile in all cases¹²². In 2000, similar experiments were performed with a GFP-expressing *E. coli*¹⁴⁰. The killing of bacteria may be caused partially by proteolytic enzymes which are present in the fore-stomach, but are more abundant in the hind stomach of the maggots as a decrease in

bacterial numbers was seen to be related to the level of enzyme activity¹⁴¹. In addition to bacterial killing in the digestive tract, maggots produce antimicrobial molecules in their excretions/secretions (ES). Many reports can be found on the killing of a broad range of microbes by ES including Gram-negative bacteria like *Pseudomonas aeruginosa*, *Escherichia coli* and *Salmonella* spp, and Gram-positive bacteria such as *Staphylococcus aureus*, *Staphylococcus epidermis*, *Listeria monocytogenes* and clinical isolates of MRSA^{123,142-145}. Unfortunately, reports either do not mention the amount of ES that was used, or they used very high amounts of ES, i.e. the equivalent of the production by more than 500 maggots in 1 hour. Obviously, the relevance of such amounts of ES would be a subject of debate. Taken together, the ES-induced altered wound pH, the ingestion and subsequent killing of bacteria in the digestive tract of the maggots, and perhaps antibacterial components within ES, could be instrumental in reducing the bacterial load in wounds.

Wound healing

The third effect of maggots on chronic wounds is induction of wound healing. Although many reports describe the appearance of granulation tissue, hardly any research has been published on how maggots induce healing. Chambers *et al* reported that, besides debridement, proteases in maggot ES degrade a variety of ECM components and concluded that enhanced lysis of the ECM could lead to increased healing¹³⁶. Prete *et al* reported enhanced proliferation of fibroblasts in the presence of different maggot preparations¹⁴⁶, whereas Horobin *et al* focused on the effect of maggots on fibroblast adhesion and migration. They reported that ES significantly reduced fibroblast adhesion to both fibronectin and to a lesser extent collagen, due to proteolytic fragmentation of the fibronectin protein surface. Based on these results, it was concluded that fibronectin fragmentation products may activate fibroblasts and enhance their migration¹⁴⁷. Using a 2D assay, they reported that fibroblast migration across fibronectin is accelerated by serine proteases present in ES¹⁴⁸. In a later study, they used a 3D assay and showed similar results¹⁴⁹. In wounds going through the normal phases of healing, these effects of ES would probably enhance healing. However, one of the characteristics of chronic wounds is the presence of enhanced protease activity which disrupts the ECM and the provisional matrix to such an extent that cell migration is impaired. Therefore, degradation of ECM components most likely does not explain the maggot-induced healing. Accordingly, Horobin *et al.* reported that 1 and 5 µg of ES/ml enhanced migration of fibroblasts (both the number of cells and distance covered) while 10 µg of ES/ml actually inhibited migration¹⁴⁹.

Scope of this thesis

In an attempt to obtain insight into the mechanisms underlying the beneficial actions of maggot therapy, we determined the effects of maggot excretions/secretions on several processes involved in wound healing. **Chapter 1** describes the various phases of the wound healing process, the possible mechanisms involved in failure of wound healing and finally the treatment of chronic wounds with medicinal maggots. The healing process in chronic wounds is often complicated by bacterial infections, especially when the bacteria reside in biofilms thus protecting them from the actions of antibiotics and the immune system. We therefore investigated the effects of maggot excretions/secretions (ES) on biofilm formation and breakdown of established *Staphylococcus aureus* and *Pseudomonas aeruginosa* biofilms. Additionally, the antimicrobial activity of ES against planktonic (free-living) bacteria is described, using two different techniques (**Chapter 2**). There was no killing of bacteria released from the biofilms and we therefore studied the synergistic effects of maggot ES and different antibiotics on the elimination of planktonic *S. aureus*, in both exponential and 'biofilm' phases and describe the effects of these antibiotics on biofilm formation itself (**Chapter 3**).

Although maggots are known primarily for debridement of chronic wounds, little information is available concerning the effects of maggots on haemostatic processes. We therefore investigated the effect of maggot secretions on blood clot formation and on the plasminogen activator induced breakdown of these fibrin clots (**Chapter 4**). Furthermore, we investigated the nature of the active component in the maggot secretions responsible for the observed effects.

The final part of this thesis focuses on the effects of maggot excretions/secretions on inflammatory cells. We report on the effects of ES on the inflammatory responses of neutrophils in reaction to the stimuli fMLP and PMA (**Chapter 5**). For this purpose, we studied chemotaxis, degranulation, H₂O₂-production and phagocytosis and intracellular killing of *Candida albicans* by these cells as well as signal transduction. Besides neutrophils, monocytes also play an important role in fighting invading bacteria. In **Chapter 6** the effects of maggot secretions on the inflammatory responses (cytokine and chemokine production, cell surface receptor expression, chemotaxis, phagocytosis, intracellular killing and signal transduction) of naïve, LPS- or LTA-stimulated monocytes are reported. Monocytes in tissues may differentiate to macrophages with a pro-inflammatory signature or to macrophages with an anti-inflammatory/pro-angiogenic signature; we therefore studied the effects of secretions on monocyte-macrophage differentiation (**Chapter 7**). The main focus of this chapter is on secretions-induced alterations in the regulatory activity of macrophages, using as a read-out the production of cytokines, chemokines and growth factors in response to LPS or LTA. In addition, the expression of various cell-surface receptors was measured.

Finally, the findings from our studies are summarised and discussed in **Chapter 8**, and a summary in Dutch can be found in **Chapter 9**.

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