



UNIVERSIDADE DE LISBOA
Faculdade de Medicina Veterinária

‘THE USE OF MANUKA HONEY AS A TREATMENT OF WOUNDS IN HORSES’

TERESA DIOGO MARQUES MAGALHÃES

CONSTITUIÇÃO DO JÚRI

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DISSERTAÇÃO DE MESTRADO INTEGRADO EM MEDICINA VETERINÁRIA

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Para a minha querida avó Teresa

Acknowledgments

Em primeiro lugar gostaria de agradecer ao Professor Doutor Luís Lamas, por toda a orientação e tempo dispensado para me ajudar na realização desta tese.

To Professor Doctor Andrew Dart for all the support during the internship and during the realization of this thesis, he was a true help emotionally and professionally, and also for helping me collect the data needed.

Gostaria de agradecer aos meus pais, por toda a ajuda emocional que me deram ao longo destes 6 anos, ao meu pai por nunca me deixar desistir de nada, e à minha mãe por ter sempre o dom de me acalmar e saber sempre o que dizer nos meus momentos de fraqueza, são o meu maior pilar.

Aos meus tios, Carminho e Tozé pela ajuda fundamental nos primeiros 3 anos de vida académica, à minha prima Sara que foi sempre um suporte emocional e sempre pronta a ajudar. Aos meus avós por todo o carinho e orgulho que demonstraram por mim ao longo de toda a minha vida.

Aos meus amigos de Coimbra, o bando dos 7 por tanto companheirismo e cumplicidade, a minha Diana, o meu afilhado, que foram as pessoas mais importantes e especiais que este curso me deu, nunca vos vou esquecer e prometo estar presente sempre que puder, levo-vos comigo para a vida.

Os meus amigos de sempre e para sempre, que foram um constante apoio, a Salé pelas noites de estudo, por me dar um ombro para chorar, e por ser a minha maior confidente, a Janica por ser como uma irmã e demonstrar sempre que se sente orgulhosa, a Inês por me dizer que vou conseguir, ao Diogo por uma amizade sempre incondicional e por não me deixar dramatizar, ao Migas pelas gargalhadas, ao Francisco pelo seu lado racional, a Carlota por me conseguir por sempre em cima, a Ana pela positividade e a Catarina por todo o apoio, foram importantes em todas as fases da minha vida e espero que continuem a ser sempre, vocês são insubstituíveis! Ao Ricardo, a melhor pessoa que eu conheço, por me ter acompanhado em todo o processo, por nunca me deixar desistir, pelo apoio incondicional a nível emocional e profissional, por me ter dito infinitas vezes que eu ia conseguir chegar até aqui, por acreditar mais em mim do que eu própria, queres sempre o melhor para mim e obrigada por me aturares nas fases de stress, que tantas foram (não sei como consegues), sou uma sortuda!

Aos amigos que apareceram mais no fim desta caminhada, a Cristina, o Serra, o Mendes, o Gonçalo, foram fulcrais na minha integração e desejo-vos todo o sucesso do Mundo.

Obrigada a todos, sem vocês não teria chegado até aqui, hoje!! Finalmente.

Resumo

O uso de mel de Manuka no tratamento de feridas em cavalos:

Os antibióticos são extremamente importantes na redução das doenças infecciosas que atualmente existem a nível mundial. No entanto, à medida que emergem as bactérias resistentes, a eficácia dos antibióticos torna-se mais limitada. A resistência bacteriana aos compostos antimicrobianos, representa uma ameaça muito séria à saúde pública uma vez que continua a aumentar exponencialmente em todo o Mundo. Para além das propriedades gerais do mel, o mel de Manuka tem uma atividade antimicrobiana diferente, na qual a sua atividade não surge devido a ter na sua composição o peróxido de hidrogénio mas sim devido maioritariamente à presença do metilglioxal. A qualidade e quantidade de evidência de que o mel de Manuka também tem capacidade de modular a resposta inflamatória inicial, através da ativação do TLR-4 nos monócitos fazendo com que haja um aumento da produção de citocinas importantes na reparação e regeneração tecidual, tem crescido.

As feridas do membro distal em cavalos cicatrizam geralmente por segunda intenção, pois a oclusão por primeira intenção não é possível devido a um variado leque de fatores. Na maioria dos casos, estas feridas apresentam um nível elevado de contaminação, uma perda de tecido avaliada de moderada a grave, uma grande taxa de retração do tecido, tempos de contração da pele mais lentos e interrupção antecipada da contração da ferida.

O objetivo de aplicar medicação tópica nas feridas é a manipulação do ambiente da mesma. O mel de Manuka quando utilizado topicamente demonstra que é capaz de modular as primeiras fases de cicatrização das feridas que fecham por segunda intenção em equinos, assim como é capaz de atuar no controlo da contaminação que caracteriza estas feridas.

Neste trabalho, foi realizada uma revisão sistemática da literatura, com o objetivo de demonstrar as evidências que existem, assim como a sua qualidade quando se fala no uso do mel de Manuka no processo de cicatrização de feridas em medicina veterinária, mais especificamente em equinos, apresentando critérios de inclusão e exclusão.

São também reportados três casos clínicos, onde o tratamento com mel de Manuka teve um papel importante e resultados benéficos de forma a validar e fornecer evidências de que é um tratamento alternativo que vale a pena ter em mente quando se lida com feridas em cavalos.

Palavras-chave: equinos; mel de Manuka; feridas; tratamento; medicina veterinária.

Abstract

The use of Manuka honey in wounds healing in horses:

Antimicrobial compounds are essentially important in reducing the global burden of infectious diseases. However, as resistant pathogens develop and spread, the effectiveness of the antibiotics is diminished. This type of bacterial resistance to the antimicrobial agents poses a very serious threat to public health, and for all kinds of antibiotics, including the major last-resort drugs, the frequencies of resistance are increasing worldwide. In addition to the generic properties of honey, Manuka honey has a non-peroxide antimicrobial activity largely attributed to methylglyoxal. Evidence that Manuka honey can also modulate the initial inflammatory response, through activation of toll-like receptor 4 on monocytes to enhance production of cytokines important in tissue repair and regeneration, have arisen.

Distal limb wounds in horses usually heal by second intention because primary or delayed primary closure cannot be accomplished. In most cases these wounds have gross contamination and moderate to severe tissue loss that would make closure difficult due to a greater wound retraction, slower rates and earlier cessation of wound contraction. They must heal completely through the process of contraction, granulation, and epithelialization. The aim of applying topical medication to wounds left to heal by second intention is to manipulate the wound environment. As a topical preparation, Manuka honey has been shown to modulate the early-period of second-intention wound healing in the horse, but this precise mechanism of action is still unclear, and has also been shown to control the contamination of the wound environment. The objective of this study is to show the actual evidences of the use of Manuka honey in the process of wound healing in Veterinary medicine, more specifically in horses by presenting a brief review of literature with inclusion and exclusion criteria, and by presenting three cases where Manuka honey treatment had a beneficial role and great outcomes, which provides evidence that it is an alternative treatment worth having in mind when dealing with wounds in horses.

Key words: equine; manuka honey; wound healing; wound; treatment; veterinary medicine.

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List of Abbreviations and Symbols

% - Percentage

spp. - species

MIC- minimum inhibitory concentration

MBC- Minimum bactericidal concentration

HBM- honey based membrane

MRSA- methicillin-resistance *Staphylococcus aureus*

MSSA- methicillin-sensible *Staphylococcus aureus*

UMF- Unique Manuka Factor

IL- Interleukin

TNF- Tumour necrosis factor

TLR4- Toll-like receptor 4

TGF-Transforming growth factor

pH- Power of hydrogen

ROS- Reactive oxygen species

COX- Cyclooxygenase

LPS- Lipopolysaccharide

MGO- Methylglyoxal

DNA- Deoxyribonucleic acid

RNA- Ribonucleic acid

DHA- Dihydroxyacetone

GH- General honey

EGT- Excessive granulation tissue

ECM – Extracellular matrix

Internship short-report

My internship took place at the Camden Equine Centre of Sydney University, Australia. It started at the 1st of October 2018 and ended 31st of January 2019, which makes a total of 850 hours of work.

Camden Equine Centre is a specialist equine hospital that is specialized in Sports medicine (performance problems, lameness and orthopaedics), Surgery, Diagnostic Imaging (with MRI, Scintigraphy, high speed treadmill with endoscopy), and Internal Medicine, as well as an Ambulatory Service.

My supervisor Professor Doctor Andrew Dart, alongside an amazing team of specialists, residents and interns guided me throughout the internship, helped me with all the theory questions and all the practice knowledge, and made these months really worth while.

During the 4 months, my internship consisted in rotating between the sports medicine, surgery and internal medicine. I had the freedom to join the cases I had most interest on, and I was able to go on ambulatory calls when I could.

In the surgeries I participated, I was able to learn a lot about the anaesthetics, with Dr. Eduardo Uquillas, able to learn about and practice asepsis before and after the surgery, and I was also able to participate in some surgeries, which for me was always the highlight of my day.

On Sports medicine, I learned a lot about MRI and Scintigraphy. Dr Robin Bell and Dr Nicole Symonds always explain everything and let students practice a lot when it comes to radiographs, ultrasound, nerve blocks, and try to help us understand all about the performance evaluation.

In the internal medicine cases, we had a lot of mare and foal care. I learned more about ophthalmology, gastrointestinal diseases and also about contagious and infectious diseases. Dr Gaby Van Galen always prompted students to discuss our clinical assessment of each case which improved my clinical knowledge.

On ambulatory calls, Dr Sara Biasutti was an outstanding teacher. During those hours in the car Dr Biasutti made us think about every possible differential diagnosis, based on the information the owners had provided prior to the call. So by the time we got to the patient we had a clear mindset about what we were going to see, and it was so much easier to understand everything we were seeing without the need to always be asking questions.

With my supervisor, Professor Andrew Dart, I had a lot of discussions about wound healing, He has a specific interest in the use of Manuka honey in second intention wound healing in horses. He introduced me to the publications he had performed, the results he had accomplished, and always advised me with respect to this thesis. Dr Dart was a great help during the internship.

Since Camden Equine Centre is a teaching hospital, I was able to spend a lot of time with the students, and participated in treatments, rounds and case presentation as one of them, which was a great learning experience as well.

From March 1st until the end of the month, I did a second externship that took place in Faculty of Veterinary Medicine of University of Lisbon in SCUE (Serviço de Cirurgia e Urgência de Equinos) with the orientation of Professor Doutor Luís Lamas, Dr Mariana Magalhães, Dr Teresa Rosa and Dr Gonçalo Silva. In this externship I got the opportunity to follow a variety of referred clinical cases, as well as improve my monitoring skills and inpatient care.

Introduction

Wounds in horses are very common, particularly wounds to the distal limb. In horses wounds usually present with tissue avulsion and contamination, which precludes primary closure so these wounds are left to heal by second intention (Bigbie, Schumacher, Swaim, Purohit, & Wright, 1991).

For many centuries, honey was used for medicinal purposes by the ancient Egyptians, Greeks and Chinese. Historical uses of honey include gastrointestinal ailments, pain relief and treatment of infections. However, it's most common use was to promote wound healing and for this purpose it was often combined with other substances such as animal fats and lint (Rogalska, 2016). In the last century the use of honey for healing decreased with the development of antibiotics (Oryan, Alemzadeh, & Moshiri, 2016). However, more recently, the emerging problem of bacterial resistance to antibiotics has seen the re-emergence of honey and other natural products. There are several properties that make honey an attractive wound dressing. It provides a moist environment, has a low pH which is favourable to the antibacterial activity, it releases oxygen from haemoglobin acting as an anti-oxidant, it draws fluid out of the wound bed because of its osmotic properties initiating an autolytic debridement amongst other properties that will be detailed further ahead (Blair, Cokcetin, Harry, & Carter, 2009; Tsang *et al.*, 2018).

The antibacterial action of most honeys is related to hydrogen peroxide. Hydrogen peroxide is generated by the enzyme glucose oxidase which is produced by the worker bees, converting glucose into gluconic acid and hydrogen peroxide. Glucose oxidase is only activated when honey is diluted, which happens when in contact with the wound exudate. This is called the peroxide activity of the honey (Cooper, 2014).

There are many different honeys, some of which have non-peroxide activity. Manuka honey derived from the *Leptospermum* trees, more specifically *Leptospermum scoparium* is native to New Zealand and is the most well-known non-peroxide honey. *Leptospermum* honeys have advantages when compared to the others honeys. They are very stable during storage (light and temperature) and when in presence of body fluids and they show no toxicity towards mammalian cells. Manuka honey has superior antibacterial action that is graded against a standard antiseptic, phenol, to provide a measure of antimicrobial activity referred to as the unique Manuka factor (UMF). The higher the UMF, the greater the antimicrobial activity (Dart, Bischofberger, Dart, & Jeffcott, 2015). This antibiotic activity is largely attributed to the presence of Methylglyoxal.

The antimicrobial effect is not the only advantage that Manuka Honey has to offer when it comes to wound healing. It has also been shown that it modulates the initial inflammatory

response through activation of toll-like receptor 4 on monocytes to enhance production of cytokines that are important in tissue repair and regeneration. It has been shown to stimulate the production of cytokines such as TNF- α , IL-1 β and IL-6, it's also believed to help enhance angiogenesis, promote autolytic debridement and accelerate re-epithelization and wound closure (Tonks *et al.*, 2003).

Compared with other topical treatments such as hydro-fiber silver or silver sulfadiazine, honey has been shown to be more effective in elimination of microbial contamination, reduction of wound area, promotion of re-epithelialization and improves the outcome of the wound healing by reducing the incidence of excessive scar formation (Oryan *et al.*, 2016). Resistance to honey has not yet been proven to exist under the conditions that rapidly induced resistance to antibiotics.

It is important to refer that there are similarities between wounds in the distal limb of horses and diabetic ulcers observed in people, including relatively poor blood supply and chronic inflammation. Reviewing the human literature may provide insight and direction into studies in the future (Tsang *et al.*, 2018).

The purpose of this review is to critically analyse the current knowledge on Manuka Honey activity in second intention wound and the healing of burns.

CHAPTER I – Literature Review

1. Skin anatomy

The skin is the largest organ of the body and serves key functions including physical protection, sensation, temperature regulation, and insulation. It is composed of 2 compartments; the epidermis and dermis. The dermal compartment consists of two regions, the papillary dermis and the reticular dermis. This compartment is composed of dense, fibroelastic connective tissue and constitutes the bulk of the skin. The strength of the skin is provided by a network of collagen fibers. The resilience of the skin is provided by elastin and glycosaminoglycans (GAGs). Collagen type I is the major collagen of the dermis whereas collagen type III comprises about 15% of the dermis. The fibroblast is the principal type of cell found in the dermis along with perivascular mast cells and tissue macrophages. Only the dermal compartment is vascularized with nutrients that reach epidermis by diffusion (Stashak & Theoret, 2008).

In the horse, the epidermis consists of five layers of keratinocytes: the *stratum basale*, the *stratum spinosum*, the *stratum granulosum*, *stratum lucidum* and *stratum corneum*.

Although 90-95% of the cell population of the epidermis consists of keratinocytes, this compartment also includes melanocytes, Langerhans cells and Merkel cells. The epidermis is attached to the dermis at the level of the basement membrane, a thin, glycoprotein-rich layer composed primarily of laminin and type IV collagen (Proksch, Brandner, & Jensen, 2008).

2. Wound healing

Wounds heal through regeneration and repair.

Regeneration is replacement of the damaged tissue with normal cells through mitosis. Repair is when the body tries to re-establish the continuity of interrupted tissues with undifferentiated scar tissue which is less biologically suitable than the tissue it replaced. The point of repair is to re-establish an epithelial cover, recover the integrity, strength, and function of the skin even when the adjacent tissues that were normal are affected by this process. Second intention repair of full-thickness cutaneous wounds is divided in 4 phases (Haemostasis, inflammatory phase, proliferative phase and remodelling phase). These phases consist in interactions between multiple cellular types, their surrounding matrix, and the soluble mediators that govern the numerous activities required to rebuild the tissue. This interaction is not static but rather in a state of constant flux, resulting in a microenvironment that is continually evolving as the wound heals (Stashak & Theoret, 2008).

2.1.Hemostasis

The first phase of second intention repair is Hemostasis/Coagulation. It begins immediately after the lesion occurs. It takes several hours and the goal is to establish hemostasis and the formation of a provisional wound matrix (De Groot, Urbanus, & Roest, 2012).

Phospholipids released by the endothelial membrane after injury are transform into arachidonic acid which mediates both vascular tone and permeability. As peripheral vasoconstriction occurs, it deprives the surrounding tissues of oxygen and nutrients that are usually carried by the blood. Hypoxia, the pH changes and increases glyucose to activate the platelets and enhance their adhesion and aggregation, which together initiate the intrinsic coagulation cascade that will end up forming a blood clot that not only seals the vessel, but it also gives strength to the wound and creates a provisional matrix that fills the space made by the wound, serving as a scaffold for migrating cells.

Inflammatory and stromal cells have special surface receptors (integrins) that recognise binding sites on the proteins present in the scaffold, which leads to the ingrowth of cells involved in healing (Herter, Rossaint, & Zarbock, 2014).

Mediators liberated by platelets and mast cells modulate vascular tone and increase vascular permeability facilitating cellular migration and diffusion of nutrients and oxygen needed to support healing. Over time the surface clot desiccates to form a scab that protects the wound from infection. This scab is, in turn, lysed by plasmin and sloughs along with dead inflammatory cells and bacteria as healing proceeds (Stashak & Theoret, 2008).

2.2.Inflammatory phase

The second phase is inflammation, also referred to clinically as the debridement phase.

The intensity of the inflammatory response is strongly correlated to the gravity of trauma and determines the extent of scarring.

It can be divided it into an early phase, characterized by recruitment of neutrophils, and a late phase, characterized by the appearance and transformation of monocytes.

Leukocytes are recruited into the injured tissues by several vasoactive mediators and chemo-attractants (Wulff & Wilgus, 2013). These signals initiate the processes of rolling, activation, tight adhesion, and, finally, transmigration of inflammatory cells through the microvascular endothelium. Chemo-attractants stimulate the release of enzymes by the activated neutrophils, expediting their penetration through vascular basement membranes. The cellular influx begins within minutes of the injury and the concentration of neutrophils at the wound gradually increases to reach a peak 1–2 days after injury.

Neutrophils act as a first line of defense in contaminated wounds by destroying debris and

bacteria through phagocytosis and subsequent enzymatic and radical oxygen mechanisms. Neutrophil migration and phagocytosis stop when contaminating particles are cleared from the site of injury. Most cells then become imprisoned within the clot, which is sloughed during the upcoming phases of repair. The neutrophils that remain die in a few days and are phagocytized by the tissue macrophages or modified wound fibroblasts, indicating the end of the early inflammatory phase of repair. Although neutrophils help create a better environment within the wound and serve as a source of proinflammatory cytokines, they are not crucial for the repair of noninfected wounds (Stashak & Theoret, 2008).

Macrophages play an important role in all phases of wound healing and control the overall process. During the early inflammatory phase, macrophages have proinflammatory functions, such as antigen presentation, phagocytosis, and the production of inflammatory cytokines and growth factors that help the wound healing. Later, during the proliferative phase of healing, macrophages stimulate proliferation of dermal, endothelial, and epithelial tissue to complete formation of the extracellular matrix (ECM), angiogenesis, and epithelialization. Macrophages can then change the composition of the ECM during the remodeling phase by delivering degradative enzymes (Delavary, van der Veer, van Egmond, Niessen, & Beelen, 2011).

After injury, once infection has been countered and repair completed, all the inflammatory cells disperse from the wound. For inflammation to resolve, each of the events that took place must stop and sometimes even reversed. Apoptosis is the universal pathway for eliminating unneeded cells in a phagocytic process. This mechanism predominates in all phases of wound repair because each phase depends on rapid increases in specific cellular populations that prepare the wound for repair (inflammatory cells) or deposit new matrices and mature the wound (stromal cells), but then must be eliminated prior to progression to the next phase of repair (Greenhalgh, 1998).

2.3.Proliferative phase:

The third phase is the proliferative phase (clinically referred as repair phase), that builds protection for the wound's surface by forming the granulation tissue, a new epithelial cover and the vascular properties for the new tissues. The proliferative phase is characterized by the appearance of red, fleshy granulation tissue, which occupies the defect. For the first 3–5 days following injury, fibroblasts, endothelial and epithelial cells rapidly invade the wound in preparation for synthesis and maturation of the matrix or for wound coverage. Granulation tissue is composed by three elements that simultaneously move into the defect created by the wound, macrophages, fibroblasts and new blood vessels. Macrophages debride and produce mediators, such as cytokines and growth factors, that stimulate angiogenesis and fibroplasia,

fibroblasts proliferate and synthesize new components of the ECM, and the new blood vessels will carry oxygen and nutrients necessary for the metabolism and growth of cells, and give the granulation tissue its red, granular appearance (Martins-Green, 2013).

This granulation bed is supposed to replace the fibrin clot with the purpose to serve as barrier to infection and at the same time works as a surface where cells are able to migrate. Fibroblasts from adjacent uninjured dermis and subcutaneous tissue are stimulated to proliferate and express integrin receptors to mediate interactions between the cells and their environment and assist them in migrating to the defect.

Migration of fibroblasts happens before advancing capillary endothelial buds but follows macrophages that have opened a path by phagocytizing debris. After the arrival of the fibroblasts in the defect, they begin to proliferate and change their function to protein synthesis. Gradually they start to replace the provisional matrix with one that is rich in collagen, under the influence of growth factors and cytokines, to increase the ratio of type I to type III (immature) collagen.

The greatest rate of accumulation of connective tissue within the wound occurs 7–14 days after injury.

The granulation tissue rich in fibroblasts is replaced by a relatively avascular and acellular scar tissue, as the capillaries in the wound regress and fibroblasts either undergo apoptosis or transform into myofibroblasts that participate in the wound contraction (Desmoulière, Redard, Darby, & Gabbiani, 1995). The wound will have a much slower gain in tensile strength as wound remodels also because the collagen content decreases. If the signal to downregulate fibroblast activity is overdue beyond a specific time point, apoptosis is permanently impaired and leads to an imbalance between collagen synthesis and degradation and the formation of excessive scar tissue. The creation of new capillaries from the ones that already exist (angiogenesis) is important to restore oxygenation and to provide the nutrients needed to the newly formed granulation tissue and that is why the microvascular endothelial cells play a key role in the proliferative phase of repair.

Angiogenesis, is a complex and dynamic process that occur in response to tissue damage and hypoxia and it's mediated by various soluble factors, the serum and the surrounding ECM. These factors are usually released during the inflammatory phase and they are angiogenic inducers, including growth factors, chemokines, angiogenic enzymes, endothelial specific receptors, and adhesion molecules, such as integrins (Liekens, De Clercq, & Neyts, 2001).

There is the need of an organized and sequential way to construct a vascular network, and that includes an higher microvascular permeability, the liberation of proteinases with local degradation of the basement membrane that surrounds the existing vessel after, then the

endothelial cells need to migrate into the interstitial space, endothelial cellular proliferation and differentiation into mature blood vessels that will be followed by the regression of the newly formed vasculature as the tissue remodels.

In the end this intense angiogenic response ends up in a density of vessel that exceed the normal capillaries in an uninjured tissue, and this is what gives the granulation tissue a red, granular appearance (Dipietro, 2013).

When the stroma has been reconstituted and this vascular supply is no longer needed, the pro-angiogenic stimuli is down-regulated and the recent capillary network involutes (Li, Zhang, & Kirsner, 2003). After this the wound will start to acquire a pale colour. Epithelialization is the process of covering denuded epithelial surfaces and is crucial for successful closure of the wound but it can only proceed once the bed of granulation tissue is formed.

While the hemostatic activities that have the purpose of creating a temporary barrier, the residual epithelium under the clot is moving centripetally in order to close of wound. The regenerative capacity of the epidermis relies on keratinocyte stem cells that reside within specific microenvironments referred to as stem cell niches. To close the defect in the epidermis, keratinocytes at the wound's edge must first loosen their adhesions to each other and to the basal lamina and develop the flexibility required to migrate over the new matrix. Once the surface of the wound is covered by epithelial cells contacting one another, further migration is inhibited by the expression, within the ECM, of laminin, a factor responsible for adhesion of epithelial cells. Although initial cellular migration does not need an increase in cellular multiplication, basal keratinocytes at the wound's margin do begin to proliferate 1–2 days after injury to restock the migratory front. In equine full-thickness wounds healing by second intention, the provisional matrix will end up being replaced by a mature basement membrane. This is a time-consuming part of the process that occurs long after the total coverage of the wound by the epithelium. This is why the neo-epidermis is fragile for so long after repair is macroscopically complete (Stashak & Theoret, 2008).

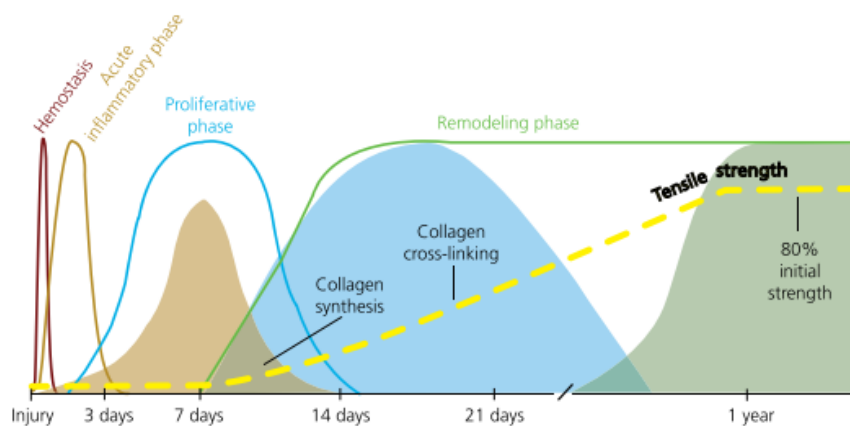
2.4. Remodeling phase:

The remodeling phase is characterized by synthesis of connective tissue, lysis, and remodeling creating scar tissue. The mature ECM is an acellular scaffold and it is made of proteins, glycosaminoglycans, polysaccharides, and water. It helps the bidirectional communication between cells and their biochemical/ biophysical environment (Wong, Gurtner, & Longaker, 2013). The remodeling phase takes place from 3 weeks to 1 year after injury, while the ECM and its components undergo changes to make sure the replacement tissue will have enough function, integrity and strength.

Successful closure of the wound by contraction is a process where both dermis and epidermis bordering a full-thickness wound are drawn centripetally over the exposed defect (Desmoulière & Gabbiani, 1988).

Wound contraction not only accelerates closure, it also provides a better cosmetic appearance and strength of the scar because proportionally less area of the wound must be covered by newly formed epithelium. Newly formed epithelium is of inferior quality. It is fragile and doesn't have normal nervous, glandular, follicular, and vascular components (Madison & Gronwall, 1992). After an initial lag phase, the skin edges retract and for 1-2 weeks the area of the wound gets bigger. After a period of rapid contraction, contraction slows as the wound approaches complete closure. Wound contraction is greater in regions of the body with loose skin than in regions where skin is under tension, such as the distal extremity of the equine limb. The new tissue remodels and randomly orientated collagen fibers start to reform along lines of stress. Points of weakness exist where the new collagen weaves into pre-existing tissue and are subject to damage when under stress. This process can take up to 2 years, during which there will be a rearrangement of the collagen fibers into a more organized lattice-like structure, under the influence of local mechanical factors that increase the tensile strength of scar tissue. The majority of type III collagen fibers laid down early in healing are replaced by collagen type I, fibers become increasingly crosslinked, and the normal skin ratio of 4:1 type I to type III collagen is reestablished. Glycosaminoglycans are steadily degraded until they reach concentrations found in normal dermis. The duration of the maturation phase depends on a variety of factors, including the patient's genetic makeup, age, location of the wound, type of injury, and duration of inflammation (Stashak & Theoret, 2008).

Figure 1- Temporal explanation of the inflammatory phases, in order to fully understand this process, and to understand that some phases occur almost at the same time. Source: Modified by Marco Langlois (Faculté de médecine vétérinaire, Université de Montréal) from Stashak & Theoret, 2014.



3. Mediators of inflammation

The process of wound repair is a set of interactive processes that involve various elements of blood, extracellular matrix, and mesenchymal cells. Recovery of structural functional properties and integrity mostly relies on soluble mediators, synthesized by cells present in the wound or within the surrounding tissue, that coordinate migration, proliferation, and synthesis of proteins by the various cellular properties involved in this process.

Cytokines are glycoproteins released by most nucleated cells and are among the most important soluble mediators regulating wound repair (Barrientos, Brem, Stojadinovic, & Tomic-Canic, 2014).

TGF β -1 peaks in first 24 hours and is a profibrotic cytokine that orchestrates the healing process, and the peak in horses is often gradual and develops over several days leading to a depressed TGF β -3 response and dysregulated fibroplasia. TGF β -3 has a reciprocal peak 5-9 days after wounding and modulates the initial inflammatory process (Bischofberger *et al.*, 2016; Tonks *et al.*, 2007).

Toll-like receptor 4 (TLR4) is believed to stimulate monocyte and macrophage activity and thereby increasing production of IL-1, IL-6 and TNF- α , that perform a major role in regeneration and repair of the skin and fastens the initiation of the inflammatory response (Tonks *et al.*, 2003).

The cytokines that play an important role in wound repair are in table 1.

Table 1: Important cytokines involved in wound repair

Name	Source	Major function
Interferon (IFN)	Monocyte and macrophage, lymphocyte, mesenchymal cell	Differentiation and maturation of hematopoietic stem cells, recruitment of inflammatory cells; mediation of epidermal proliferation; promotion of myofibroblast differentiation and wound contraction
Interleukin (IL)	All nucleated cells, in particular macrophage and lymphocyte	Proinflammatory; release of other cytokines; inhibits fibrosis
Tumor necrosis factor (TNF)	Macrophage, lymphocyte, mast cell	Proinflammatory; enhances epithelialization, angiogenesis, and remodeling
TGF- α	Macrophage, epithelial cell	Remodeling and angiogenesis
TGF- β	Platelet, lymphocyte, mast cell, monocyte and macrophage, endothelial cell, epithelial cell, fibroblast	Chemotactic to inflammatory cell and mesenchymal cell, fibroblast proliferation; protein synthesis; ECM deposition; wound contraction.

4. Bacterial resistance – the reality

The development of antibiotics saw a decrease in the use of natural alternatives like honey. Nowadays most antimicrobials when tested have at least three different types of resistant bacteria. At the same time there is not enough research into producing new antimicrobials because the financial benefits to the pharmaceutical industry are not returned because resistance of new antibiotics is realized. Interest in ancient remedies, like plants and plant-based products, including honey are being re-evaluated (Mandal & Mandal, 2011).

It has been suggested that the risk of bacteria developing resistance to honey will be low if high concentrations are used clinically. High levels of antimicrobial agents can effectively inhibit viable bacteria before resistant strains emerge. Unlike some of the biocides, high amount of manuka honey is not cytotoxic (Cooper, Jenkins, Henriques, Duggan, & Burton, 2010).

The fact that Manuka honey works has an antimicrobial due to more than one factor, and also affects the bacteria in different ways, is one of reasons believed to explain the non-resistance of honey (Oryan *et al.*, 2016). Future research is needed in order to enhance the outcome of honey, there is still a gap of knowledge when it comes to know how all the properties work.

5. Physical characteristics of honey in general

5.1.pH

Honey is acidic and has a pH of around 3.2-4.5. This low pH is due to the formation of gluconic acid. During the dilution of honey, glucose oxidase catalyses glucose into gluconic acid and hydrogen peroxide where the gluconic acid results in acidity.

It is well known that topical acidification of wounds promotes healing by increasing the release of oxygen from haemoglobin in capillaries and because it is unfavourable for bacterial growth (Oryan *et al.*, 2016).

In addition, this pH is less favourable for protease activity. Protease contributes to poor tissue healing by damaging the extracellular matrix and destroying cytokines and growth factors. So by reducing the destruction of the matrix needed for tissue repair, it contributes to wound contraction.

Acidification also assists in the antibacterial action of macrophages and prevents the ammonia produced by the bacterial metabolism from harming body tissues (Molan & Rhodes, 2015).

5.2. Sugar/Osmose

Honey is made of 80% sugars, 18% water and a complex mixture of amino acids, peptides, arabinogalactan proteins, organic acids, polyphenols, carotenoid-like substances, flavonoids, vitamins and minerals. The high sugar content produces a high osmotic gradient that leads to bacterial dehydration and shrinkage of the cell wall. This high osmolarity also initiates an influx of fluid, lymph and nutrients into the wound bed, creating a moist environment rich in nutrients. These properties promote an autolytic wound debridement and enhance tissue healing (Tsang et al., 2018).

High osmolarity solutions such as honey, tie up water molecules. Honey, as a viscous fluid, provides a protective barrier and prevents cross-infection of wounds. When honey is topically applied over a wound, due to its high osmolarity, bacteria have insufficient access to water for growth and development. Consequently, the microorganisms become dehydrated and eventually die. Also osmotic pressure from honey draws out lymphatic fluid from the subcutaneous tissue to wound surface, which aids in removal of debris, necrotic and devitalized tissues (Oryan *et al.*, 2016).

The dilution of the honey by the wound exudates could be a problem, but it is believed that if the honey does not become too diluted it still inhibits the growth of bacteria (Minden-Birkenmaier & Bowlin, 2018).

5.3. Anti-oxidant

Chronic wounds are highly oxidizing environments because of release of reactive oxygen species (ROS) from persistent neutrophils and macrophages infiltration (Majtan, 2013).

There are several components that can also contribute to the antioxidant activity of honey such as, flavonoids, phenolic acids, catalase, peroxidase, ascorbic acid, and carotenoids.

The presence and concentrations of these antioxidants differs between the many types of honey and therefore the antioxidant properties.

Woo et al. found that chrysin, a natural flavonoid found in many plant extracts, honey, and propolis acted to inhibit cyclooxygenase-2 (COX-2) gene expression in LPS- stimulated cultured macrophages. This effect was mediated through inhibition of the binding activity of nuclear factor IL-6 (Woo, Jeong, Inoue, Park, & Kwon, 2005).

A decrease in neutrophil superoxide production has been reported (Van den Berg *et al.*, 2008). So, the antioxidant activity of honeys was mostly attributed to the inhibition of ROS formation either by inhibiting the respiratory burst of neutrophils or by direct ROS scavenging. A dose-dependent reduction in the production of superoxide from human neutrophils by some honeys

did not correlate with the level of known honey-based phenolic compounds, which are well-known free radical scavengers. This indicates that the anti-oxidant activity of honey is most probably caused by inhibition of the respiratory burst of neutrophils (Majtan, 2013).

5.4.Methylglyoxal (MGO) vs Hydrogen Peroxide

Hydrogen Peroxide is formed in the honey by an enzyme called glucose oxidase (Mavric, Wittmann, Barth, & Henle, 2008; Song, Salcido, & Erdman, 2011).

This enzyme is produced by worker bees and is found in all types of honey in varying concentrations and it is responsible for oxidizing glucose into gluconic acid and releasing hydrogen peroxide.

Hydrogen peroxide is only detected in diluted honey because dilution leads to activation of glucose oxidase. The generation of hydrogen peroxide is greatest when honey is diluted 30-50%, so in the presence of wound exudate the antimicrobial activity of hydrogen peroxide, which is one of the most used disinfectants, may contribute significantly to the antibacterial activity in some honey varieties (Cooper, 2014; Tsang *et al.*, 2018).

Other factors that can contribute to the variability of the activity and concentration of hydrogen peroxide can be because of the plant species, environmental conditions and entomological factors, including age of the bee and foraging patterns (Tsang *et al.*, 2018).

It is known that high concentrations of catalase present in body fluids, inhibits the activity of hydrogen peroxide. However it has been shown that honeys derived from some plant sources have superior antimicrobial activity even in the presence of this enzyme.

This has been identified has the non-peroxide activity. Manuka honey is a non-peroxide honey and this degree of activity is measured and reported as the Unique Manuka Factor (UMF) (Tsang *et al.*, 2018).

MGO is an organic compound found in manuka honey and it has been shown to be responsible for the majority of the antimicrobial activity. The mode of action is still not fully elucidated but from what it's known it acts through its capacity to involve a combination of enzymatic and non-enzymatic processes that involve the capacity to interact with macromolecules such as DNA and RNA (Irish, Blair, & Carter, 2011; Tsang *et al.*, 2018).

MGO comes from a non-enzymatic conversion of a chemical found in high concentrations in the flower of the manuka tree, dihydroxyacetone (DHA). DHA has recently been shown to limit the production of hydrogen peroxide by inhibiting the enzyme glucose oxidase (Tsang *et al.*, 2018), so hydrogen peroxide does not contribute to the antimicrobial activity of manuka honey (Oryan *et al.*, 2016).

5.5.Others

It has been suggested that the antibacterial factors within honey are likely to interact together, thereby creating a synergistic effect and a regulating activity of other chemicals (Molan, 2008). Another characteristic of honey dressings is that it reduces pain. The pain in wounds results from the nerve endings being sensitized by prostaglandins produced in the process of inflammation, as well from the pressure on tissues resulting from oedema. Because of this high sugar content, honey also prevents pain on dressing changes as, by mobilizing the oedema from the surrounding tissues, it keeps the wound surface moist (Simon *et al.*, 2009).

6. Honey in wound healing

The honey we use generally in wound healing is a Medical grade honey.

Honey has been considered a prized and exceptional remedy with a lot of different properties; it has a clinically proven effect in wound healing, it eliminates pathogens from wounds and it even provides an appropriate moist environment for effective healing (Majtan, 2013).

Variations in the type and level of antimicrobial activity in honey is associated with the floral source. However, while some floral sources appear to be associated with particular levels of hydrogen peroxide activity, variation in this activity among honeys from within the same floral species has also been observed. This may be due to the geographical location of the floral source and the prevailing environmental conditions, which affect the physiology of the floral species, or bee-related factors, such as age or colony health, which may affect the production or activity of glucose oxidase. Honeys with non-peroxide antimicrobial activity on the other hand, are more closely associated with floral source, being generally derived from *Leptospermum* species (Irish *et al.*, 2011).

The honey this review studied in detail is the one that has its origin in the *Leptospermum scoparium* commonly known as Manuka bush.

7. Manuka honey

Manuka honey is a non-peroxide type of honey, and it's produced from the manuka tree (*Leptospermum scoparium*) found in New Zealand (Shah, 2017).

Honey derived from the manuka tree has been found to display superior antimicrobial activity compared to many other honey varieties. As a result, the increasing public awareness of its health benefits, has seen the cost of raw manuka honey increase more than 10-fold over the past 20 years (Stephens *et al.*, 2010).

Manuka honey is not affected by catalase, and it has a unique antibacterial activity. This activity is due to the methylglyoxal, which forms by spontaneous conversion in ripened honey from its precursor substance dihydroxyacetone that is found in the manuka nectar. Methylglyoxal on its own is a cytotoxic substance, so it has been raised that manuka honey may contribute to delayed wound healing, however it seems that, the combination and ratio with other components in the manuka honey neutralizes any toxic effects (Majtan, 2011).

One important advantage about manuka honey and about the non-peroxide antimicrobial activity honeys in general, is that their activity is not destroyed by the enzyme catalase present in body fluids. This, together with the fact that it's not affected by the gamma-radiation, allows the honey to be sterilized for medicinal use (Irish *et al.*, 2011).

Figure 2 and 3: Photographs of a Standardbred horse with wounds along its flank and down the back leg caused by a clostridial myositis after injection with a contaminated needle. (Courtesy of Professor Doctor Andrew Dart).



Clinical case of a horse presented with extensive subcutaneous emphysema, febrile, depressed and urinating bloody stream of urine. Under local anaesthesia the affected area was sliced open with a scalpel in order to open the area to oxygen. Horse was placed on penicillin and fluids. Wounds were treated with Manuka honey daily. Photographs taken several weeks after horse being in, and treatment was continued throughout healing. Horse went back to racing.

7.1.UMF rating

The so-called Unique Manuka Factor is a trademarked grading system placed on the label of commercially available Manuka honey by licensed producers in New Zealand. The UMF rating assures the purity and quality of the product by representing a similar zone of growth inhibition in a radial diffusion assay with *Staphylococcus aureus* when compared with a known concentration of an antiseptic (phenol) solution. Each individual batch of manuka honey is tested for antimicrobial activity, however, it is worth noting this testing does not demonstrate precisely which components contribute to this property. In general, a UMF rating 10-15 is required for the batch to be considered therapeutically useful, whilst a UMF 16-30 has superior activity with high antimicrobial efficacy. A UMF rating 5-9 has minimal antimicrobial activity and is not recommended for therapeutic use as an antimicrobial agent, whilst a UMF 0-4 has no detectable antimicrobial activity (Tsang *et al.*, 2018).

Tsang *et al.* in 2017 compared the effects of topical application of UMF20 and UMF5 manuka honey with a generic multi-floral honey. The results showed that when using the UMF20 the total healing time is reduced when compared to UMF5 and general honey (GH). Furthermore, healing times of wounds treated with GH and UMF5 were not significantly different. In this same study, it was concluded that application of UMF20 to wounds for 12 days showed a longer total healing time compared to using UMF20 until the wound is completely healed (A. S. Tsang *et al.*, 2017).

7.2.What's the difference between MGO and UMF on the label of the commercial products?

Commercial available manuka honey products are often labelled in different ways, so it is important to understand what it means. The UMF factor is the internationally registered and recognised trademark. It is produced by companies that measure the antibacterial activity of a batch of honey against phenol, a standard antiseptic. The higher the UMF, the greater the antibacterial efficacy. There are products labelled with an MGO concentration and others with a UMF. Products showing MGO concentration have measured the weight by volume of MGO in that batch of honey. While it is accepted that an increasing concentration of MGO is related to the antibacterial activity, the antibacterial activity has not been tested. Studies have shown that taken out of Manuka honey, the activity of MGO can vary, suggesting that other factors in Manuka honey can affect the antimicrobial activity of Manuka honey (Tsang *et al.*, 2018). So, while concentration of MGO in any batch of Manuka honey broadly correlates with antibacterial activity only honey with the registered international trademark provides assurance of the medical grade of Manuka honey.

7.3.Effect on biofilms

Bacterial biofilms are known to cause difficult healing of wounds in both humans and horses due to the ability of biofilms to interfere with the activity of many antibiotics. Manuka honey has been shown to inhibit the formation of biofilms and cause detachment of established biofilms, although the exact mechanism of action is not fully known. Exposure of bacteria to manuka honey *in vitro* has demonstrated downregulation of genes coding for surface-binding proteins important in biofilm formation, virulence, and cell to cell communication. Low concentrations of manuka honey may prevent biofilm maturation by disrupting cellular communications, whilst higher concentrations of manuka honey are able to penetrate and detach established biofilms. The methylglyoxal in manuka honey appears to play a critical role in the burst of biofilms but it is not totally responsible for this property (Tsang *et al.*, 2018).

The antibacterial component of manuka honey is a small water-soluble molecule that diffuses easily, which explains why manuka honey has also exhibited efficacy against bacteria contained in biofilms. Prolonged chronicity of wounds can be attributed to wound colonization that develops into a biofilm in which the bacteria stay protected by the matrix of the biofilm. These bacteria cannot be cleared by the host immune system and show resistance to both systemic and topical antimicrobial agents. This may explain why antibiotics are of limited use in treating chronic wounds. Manuka honey at a concentration of 40% has been found to give significantly reduced biofilm mass with *in vitro* testing of clinical isolates of *P.aeruginosa* that had developed into a biofilm (Cooper, 2009). Similar findings were seen in studies with *Streptococcus pyogenes* (Maddocks, Lopez, Rowlands, & Cooper, 2012), methicillin-resistant *Staphylococcus aureus* (MRSA) (Alandejani, Marsan, Ferris, Slinger, & Chan, 2009), where it was found biofilms were sensitive to manuka honey treatment (Molan & Rhodes, 2015).

7.4.Effect in gram (-) and gram (+)

The mode of action of Manuka honey differs with the different types of bacteria that it's dealing with. Exposure of gram-positive organisms to MGO leads to the downregulation of autolysin, an enzyme involved in cell division, and the cleavage of bacterial cell wall components. On the other hand, when exposure of gram-negative bacteria to MGO occurs, it appears to lead to altered gene expression of proteins involved in the structural stability of the cell wall and cell lysis (Dart *et al.*, 2015). A decrease in virulence factors of bacteria has also been observed following treatment with manuka honey, including downregulation of flagella-associated proteins, inhibition of siderophore formation, and reversal of antibiotic resistance (Tsang *et al.*, 2018).

8. Effect in malodour

The deodorisation of offensive odour from wounds is an expected consequence of honey's antibacterial action. The malodour is due to ammonia, amines and sulphur compounds, which are produced when infecting bacteria metabolise amino acids from proteins in the serum and necrotic tissue in a wound. The rapidity of honey's deodorising action is probably due to the provision of a rich source of glucose, which would be used by the infecting bacteria in preference to amino acids, resulting in the production of lactic acid instead of malodour compounds (Molan, 2014).

9. Storage

Processing and storage conditions, including heat, light and catalases produced by damaged cells, pollen and some bacteria can inactivate hydrogen peroxide which leads to a variable antibacterial activity of some types of honey under different conditions (Dart *et al.*, 2015).

In contrast, the levels of DHA in stored Manuka honey slowly decreases over time as it is converted into MGO, which subsequently increases at a similar pace when stored at 37°C. The concentration of MGO in stored honey plateaus after 3-4 months and maintains a consistent ratio of DHA to MGO of 2:1 (Oryan *et al.*, 2016; Tsang *et al.*, 2018).

10. Can we use General food honey as a wound dressing as well?

Recent studies made at the University of Sydney, showed that generic honey from a supermarket was no more effective than isotonic saline to treat experimental contaminated wounds (Tsang *et al.*, 2017).

Within the environment there are spores that pervade, existing in the soil, air, dust and raw agricultural products, including *Clostridium spp.* Clostridial spores are a real concern in the anaerobic environment of some open wounds. It is recommended that honey used in wound healing be sterilized. Heat can change the properties of some honeys to eliminate spores. Medical grade honey is often gamma-irradiated to inactivate spores such as those from *Clostridium spp.* This does not have a detrimental impact on the antibacterial activity of honey (Simon *et al.*, 2009).

11. Recommendations on using a Manuka honey wound dressing

The recommendations and approaches on how to use honey as a dressing depends on the type of wound we're dealing with, the site of the wound, the level of contamination that is present, and among other factors, the way the commercial product is made.

Contaminated or severely traumatized wounds should be surgically debrided to remove necrotic tissue and debris to optimize healing.

The application of Manuka should start as soon as possible in order to achieve the best outcome, preferably within the first 24 hours after wounding. The honey should be at a normal room temperature for an easier application.

When bacterial contamination and tissue trauma is present, manuka honey with a UMF ≥ 15 should be used (Bischofberger *et al.* 2013). However, it's important to note that as the UMF of manuka honey increases so does the cost.

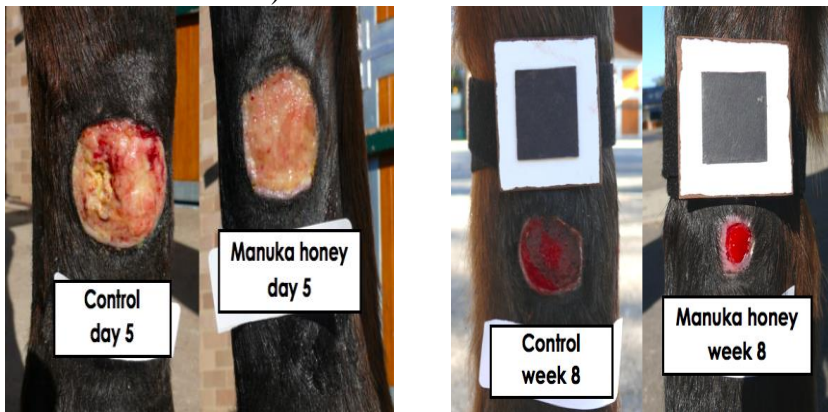
A study by Dart (2013) found that an effective way to decrease the costs, is to dilute the manuka honey to 66% together with 34% of sterile water-based gel. Wound healing was found to be unaffected by dilution (Bischofberger *et al.*, 2015).

Bandages can have as many advantages as they can have disadvantages. Bandages will improve the contact time between the honey and wound and may be useful in the early stages of wound healing. However, ongoing bandaging may be associated with high incidence of extra granulation tissue (EGT) development, and in this case the EGT should be excised as it arises, and the application of honey continued (Carr, 2003). If a bandage is applied, the duration of application should be kept to a minimum, preferably under 12 days, to reduce the chances of EGT (Bischofberger, Dart, Perkins, & Dart, 2011).

The first application will have to be changed sooner than the ones to follow, because the amount of exudate will be greater due to the osmotic action of the honey happening at the same time as the peak activity of the inflammatory phase that causes a high level of exudate (Kennedy, 2018). On the following applications, as long as the dressing still has Manuka honey available and in contact with the wound and if there is no exudate soaking through the overlying bandage, it can be left in place for a maximum of 5 days, after that a change of bandage is recommended (Hollis, 2016). In open wounds, applying a thin film 2-3 times daily, may be the best approach, because the contact time and the efficacy are both optimized.

According to the duration of the treatment there are also different opinions. A study was undertaken to try to understand the outcomes between application of manuka for 21 days, and the application until the wound healing is complete. It showed that a wound heals faster if the treatment continues until the healing is complete (Dart *et al.*, 2015).

Figure 4 and 5: Photographs showing the action Manuka honey had in the treatment of one wound compared to a wound without treatment in different time lines. (Courtesy of Professor Doctor Andrew Dart)



CHAPTER II – Use of Manuka honey to treat wounds in horses – state of the art and case reports

1. Aims

The aim of this literature review was to evaluate the quality of the available data on this subject, to identify gaps in current knowledge and ensure there is justification for future research on the subject and to add more information on the matter to this dissertation.

The aim of the clinical cases presentation is to report three real cases where Manuka honey was used as the major treatment, and to show the practical application and the outcome that I was able to follow part of the process during my internship, and observe all the theory I had studied put into actions.

2. State of the art about of Manuka honey in wound healing in Veterinary Medicine using inclusion and exclusion criteria

2.1. Search strategy and Data sources

This search was conducted in accordance with the evidence-based guidelines for systematic reviews set forth in the PRISMA statement. Published studies were identified from 2009 to 2019, that included information about the use of Manuka honey in wounds in Veterinary Medicine, more specifically in equine practice. The search was conducted in 2 relevant databases (PubMed and ScienceDirect). In each database, the following search terms were used: “wound”, “wound healing”; “veterinary”, “equine”, “horses”; “Manuka honey”. Each individual search consisted of a combination of the 3 terms connected by “AND”. Each individual search within a database was then combined using “OR” to account for duplication. The electronic search was complemented with a hand-search in references of review articles and book chapters

2.2. Selection of articles

The abstracts of articles were selected by applying the following inclusion criteria: (a) research studies and case reports; (b) published studies; (c) written in English language; (d) including research using Manuka honey in wounds; (e) studies related to Veterinary medicine; (f) studies with important information for this review with relevant results.

Because a large number of new studies have been conducted in the last few years, the information before 2009 was not included.

By analyzing the titles of the publications, I was able to determine that most studies did not meet the inclusion criteria because they did not focus specifically on the topic.

Nevertheless, all abstracts were analyzed, and full-text versions of those that met the inclusion criteria and presented original research on the topic were obtained and assessed. Data from those studies were extracted into an evidence table.

2.3. Screening process

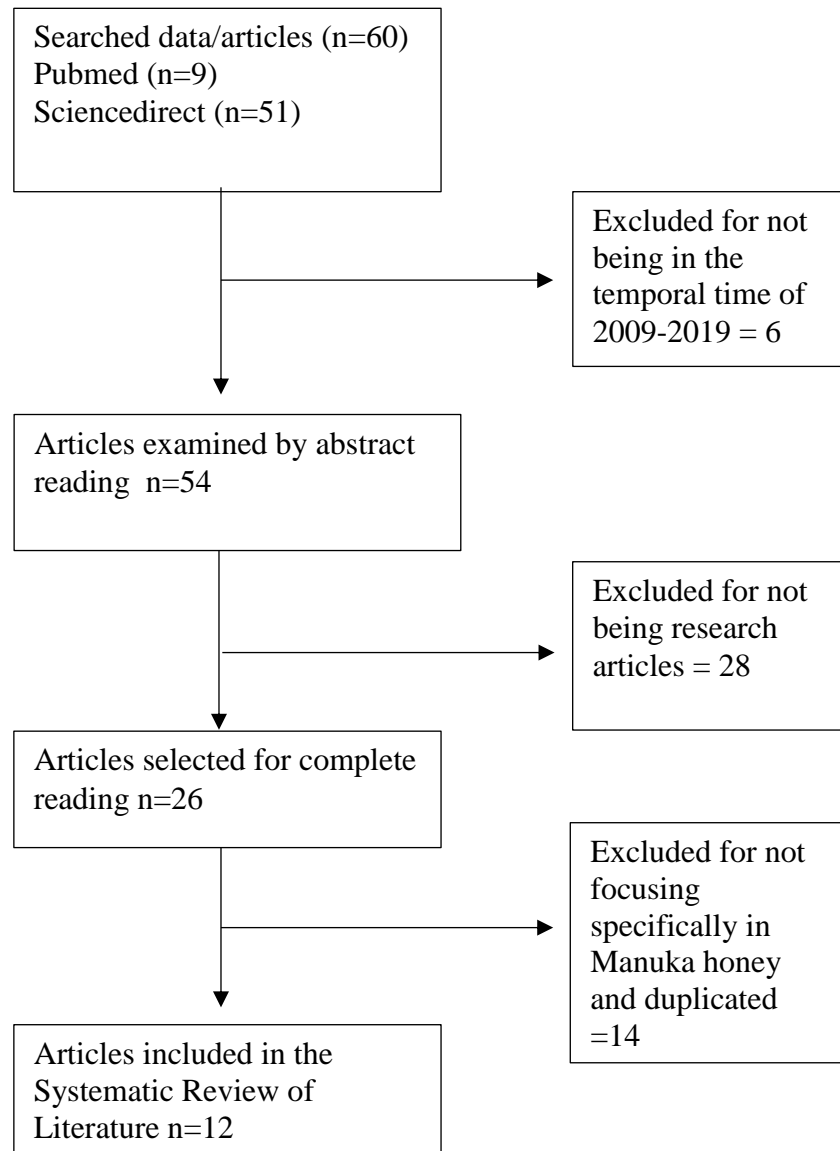
Articles that did not meet the inclusion criteria or met the exclusion criteria were assessed first by their title and secondly by their abstract and were removed. The full text was then obtained, if possible. Articles were accessed through the internet if access was available from the Faculty of Veterinary Medicine of the University of Lisbon. If an article was unavailable online or at the Faculty of Veterinary Medicine Library, an attempt to retrieve it by electronic contact with the publishing journal was made.

3. Results

3.1. State of the arts – search results

A visual summary of the study selection process is presented in Figure 1. The search strategy yielded 56 studies as potentially relevant in the initial searches, of which 12 met the inclusion criteria and were included in the present review. Data from each eligible study were collected and summarized in tabular format (see Table 2 and Table 3). Key information was collected on the sample characteristics, methods, outcomes and if control groups were present. The data were then discussed to ensure congruence of the information extracted.

Figure 6 : Flow diagram of the study selection process.



3.2. Overview of the included studies

Of the 12 papers included in the present review, most studies were published in veterinary journals. The final 12 papers consisted of research projects and case reports. After analyzing the papers, it was decided to divide the results in two groups: *in vivo* and *in vitro* according to the type of research performed and reported. The main results are presented in table 2 and table 3.

Table 2: Research collected data of the use of Manuka honey *in vivo*

Authors	Sample	Control groups	Anti-inflammatory activity	Anti-microbial activity
Bischofberger, <i>et al.</i> 2016	10 Standardbred horses	Yes	Yes, assessed with biopsy	No activity shown on bacterial counts
Hollis, G. 2010	One Cat, one Dog, one Horse	No	Yes, macroscopically	Not assessed
Herdan, <i>et al.</i> 2012	Three Thoroughbred horses	No	Not assessed	Not assessed
Giusto, G. <i>et al.</i> 2017	36 Sprague-Dawley rats	Yes	Yes, macroscopically and pathology analysis after euthanasia	Not assessed
Bischofberger, <i>et al.</i> 2016	Eight Standardbred horses	Yes	Yes, biopsied for histopathology	Not assessed
Bischofberger, <i>et al.</i> 2013	Ten Standardbred horses	Yes	Yes, macroscopically	Not assessed
Bischofberger, <i>et al.</i> 2011	Eight Standardbred horses	Yes	Yes, by repeated measurements of the wound area	Not assessed
Tsang, <i>et al.</i> 2017	Eight Standardbred horses	Yes	Yes, by repeated measurements of the wound area	Not assessed

Table 3: Research collected data of the anti-microbial effect of Manuka honey when tested *in vitro*

Authors	Sample	Method	Control groups	Anti-microbial activity
Tramuta, <i>et al.</i> 2017	60 Manuka HBMs and 60 Honeydew HBMs.	Evaluating survival of bacteria strains after incubation for 1,3,6 and 24h at 35°C.	Yes	Yes
Carnwath, <i>et al.</i> 2014	29 honey products, only 11 assessed	Evaluating the honey plates after 16-24h culture for the presence or absence of visible colony formation.	Yes	Yes
Almasaudi, S.B. <i>et al.</i> 2017	Five types of honey	Mueller–Hinton Agar with honey added was cultured with MRSA and MSSA and evaluated for inhibition of bacterial growth.	Yes	Yes
Grego, <i>et al.</i> 2016	27 types of honey	Agar well diffusion method.	Yes	Yes

3.3.Reviewed studies

Eight studies (66.7%) provided information on how Manuka honey acts as a wound healing dressing using animals (*in vivo*) and addressing the way Manuka acts as an antimicrobial and anti-inflammatory agent. Some studies compared Manuka honey with other types of honey, other studies compared the effect of different UMF rates of commercial medical Manuka honey. Five of these studies were performed in horses using their limbs as the model to create surgical wounds (Bischofberger et al., 2016b, 2015; Bischofberger et al., 2011, 2013; Tsang et al., 2017). Some studies replicated contaminated wounds to try to recreate a real-life scenario of contamination by applying fresh feces of a horse in the wounds for 24 hours and leave other wounds uncontaminated to compare the action of Manuka honey in contaminated “versus” uncontaminated wound environment. One study used rats for the trial and wounds were created on the dorsum and aimed to observe which dressing had a better outcome (Giusto *et al.*, 2017). There were two case reports, one about three real life cases of different animal species, where Manuka honey was included in the therapy (Hollis, 2014), and one that consisted of three real life cases of horses that were presented with septic synovitis and Manuka was used in two of the three cases only as a wound healing, this study was not of great relevance for this review (Herdan *et al.*, 2012). None of these two presented control groups.

The remaining four studies (33.3%) worked with Manuka honey *in vitro*, using different techniques (Almasaudi et al., 2017; Carnwath, Graham, Reynolds, & Pollock, 2014; Grego et al., 2016; Tramuta et al., 2017). These studies aimed to make conclusions about the antimicrobial activity of Manuka honey comparing the different UMF Manuka honey and comparing these with other types of honey, against common clinical pathogens in veterinary medicine. Some of these studies also searched for the level of bacterial contamination of the honeys, and the possible antibiotic residues that might be in the honeys.

3.3.1. *In vivo* studies

Eight studies were *in vivo* (66.7%), and one of them did not have relevant results to this review, as said before (Herdan *et al.*, 2012).

The majority of the studies were able to show a positive outcome on the use of Manuka honey, described the methods that were used, and the outcomes were reported clearly. There are two major variables being tested in the studies, the anti-inflammatory action and the anti-microbial action of Manuka honey.

In all the seven relevant studies (100%), as referred in the table 2, Manuka honey showed an efficient anti-inflammatory activity during the treatment, but more specifically during the first

three weeks (21 days), acting more successfully during the initial phases of inflammation, assessed by the observation of development of a healthier bed of granulation tissue, increased angiogenesis, fibrosis, collagen organization, in the first 10 days, and by moisturizing the wound throughout the treatment enhancing the debridement activity (Giusto *et al.*, 2017; Tsang *et al.*, 2017). In some of the studies (50%) the total healing rate of the treatment with Manuka honey did not differ from the untreated control wounds, the hypothesis is that the natural occurring wound environment was not recreated successfully and that some studies had a complicated level of interpretation due to the several levels of treatment (Bischofberger *et al.*, 2016, 2015; Bischofberger *et al.*, 2011).

In four studies (50%) the anti-microbial activity of Manuka honey is thought to have a successful outcome, and the studies where antimicrobial activity was not shown, some limitations were hypothesized.

In one study (12.5%), the treatment with Manuka honey was assessed with half the horses bandaged and the other half unbandaged, to see if this would cause any change in the healing pattern. Bacterial counts in the unbandaged wounds increased between day 2 and 7, irrespective of the treatment. Histologically, in the current study there were few differences between bandaged and unbandaged wounds in the first 10 days following wound creation, but where differences were identified they consistently reflected higher fibrosis and collagen organization and higher epithelial hyperplasia scores in bandaged wounds (Bischofberger *et al.*, 2016).

In another study present in this review (12.5%), the time of the treatment was assessed along with other variables. The aim was to see if there were more advantages in performing a short or long-term treatment with Manuka honey. In some wounds the treatment stopped after 12 days, and in others continued throughout healing. The results were clinically insignificant, but still supported that extended treatment with Manuka honey increased the beneficial effect, even if the fastest healing rate happens between day 7 and 21. These results supported should be evaluated carefully since these results cannot be extrapolated because these wounds were surgically created and do not replicate the naturally occurring wounds that often are larger and associated with substantial tissue trauma. However, in this study the results conclude that the most beneficial time to apply Manuka honey is in the first 3 weeks after wounding (Bischofberger *et al.*, 2013).



Figure 7-
Photograph of
wounds being
created surgically



Figure 8-
Photograph of
wounds
immediately
following the
surgical creation

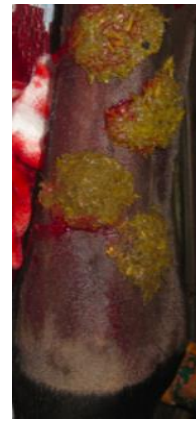


Figure 9-
Photograph of
wounds
contaminated with
feces



Figure 10-
Photograph of
wound after being
biopsied.



Figure 11- Photograph
of the method of
measurement used to
assess the size of the
wounds.



Figure 12- Photograph of
the method of
measurement used to
assess the size of
contaminated wounds.

(Figures 7,8,9,10,11 and 12 of courtesy of Professor Doctor Andrew Dart)

3.3.2. *In vitro* studies

Four studies were made *in vitro* (33.3%), and the aim of all of them was similar, to assess the antimicrobial activity of Manuka honey against the most common bacteria, while comparing with different types of honey (medical and locally produced). In 100% of the *in vitro* tests, Manuka honey performed successfully against the tested bacteria. Three studies (75%) showed that Manuka honey was the best performing honey (Almasaudi *et al.*, 2017; Grego *et al.*, 2016; Tramuta *et al.*, 2017), and one study (25%) concluded that Manuka was not the best performing honey, but still presented effectiveness at inhibiting microbial growth at low concentrations (Carnwath *et al.*, 2014). One of these studies assessed the activity of Manuka honey against MRSA and MSSA and the results showed a high bactericidal activity at low concentrations, while other honeys only made a bacteriostatic effect on these bacteria (Almasaudi *et al.*, 2017). One of the studies also assessed the MIC and MBC and the results were in favor of Manuka honey in comparison with the other honeys (Grego *et al.*, 2016).

4. Case reports

Three cases were included in this study, two cases that I was able to follow in part during my internship in Camden Equine Centre, and one case was provided by Professor Doctor Andrew Dart (case 3). All the information about the horses was provided by my supervisor, Professor Doctor Andrew Dart, as well as the photographs presented in the results section.

4.1. Case 1

This is a case of a horse with a severe right upper leg wound. The horse went through the bottom of a float. The floor rusted through and the horse was dragged along the bitumen. The animal was immediately brought in for euthanasia, but the owners were told the wound could heal. The horse was hosed and treatment consisted in daily application of Manuka honey throughout healing. Wounds were hosed before applying Manuka honey. The wounds were macroscopically assessed every day, and photographs were taken every week along the treatment to record the effect of Manuka honey.

Figure 13- Case 1: Photograph of wound few days after wounding. (Courtesy of Professor Doctor Andrew Dart)

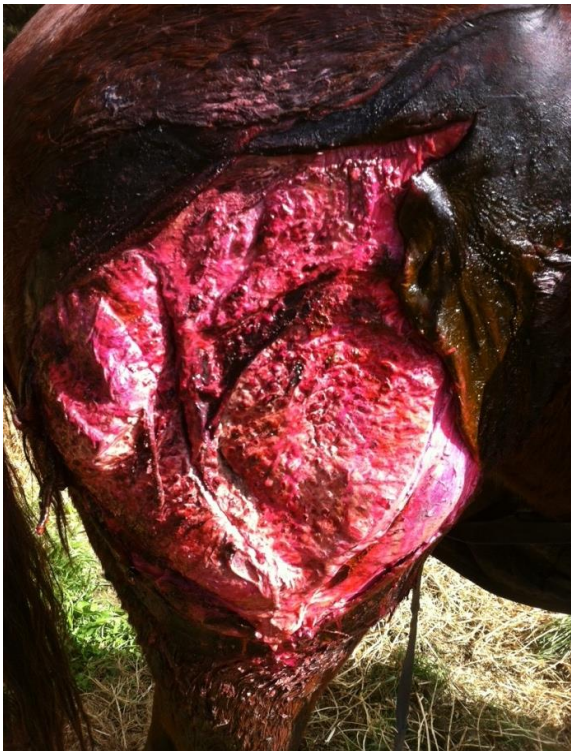


Figure 14 – Case 1: Photograph of wound one week after treatment with Manuka honey. (Courtesy of Professor Doctor Andrew Dart)



Mummified tissue can be notice around the edges of the wound. The tissue was kept in place for physically protection of the wound. At this stage the wound had a fly strike and a treatment with insecticide was started intermittently.

Figure 15 – Case 1: Photograph of wound after two weeks of treatment with Manuka honey.
(Courtesy of Professor Doctor Andrew Dart)



At this stage the wound showed a good blood supply and a formation of an healthy granulation bed.

Figure 16 – Case 1: Photograph of wound three weeks after daily application of Manuka honey.
(Courtesy of Professor Doctor Andrew Dart)



Seven days later we can observed the wound starting to dry out and the mummified tissue detaching from the wound.

Figure 17 – Case 1: Photograph of wound 4 weeks after daily application of manuka honey. (Courtesy of Professor Doctor Andrew Dart)



At this point, epithelialization of edges is evident and the wound is noted to be contracting. The horse went home at this time to continue the treatment. A picture of the wound 3 months after wounding was observed and the wound had fully contracted and healed completely. The horse went back in work as a camp drafting horse.

4.2. Case 2

The second case is a case of a Quarter-horse with a severe injury involving the left posterior leg possibly injured after running against a fence. The extensor tendon function was completely lost.

Left posterior lower limb was presented with a great tissue avulsion, contamination and closing by first intention was not possible. The horse was presented at the hospital at the day of wounding, and could not stand on the left leg due to the extensor tendon function being completely lost.

The lower limb was carefully cleaned and clipped. The horse was anaesthetised and wound was surgically debrided. A bandage was placed and topical Manuka honey treatment was started on day 1, and applied at bandage changes every 2-3 days.

Figure 18 – Case 2: Photograph of day 1 of wounding. (Courtesy of Professor Doctor Andrew Dart)



In this photograph we can observe the severe tissue avulsion and the horse not able to stand in the injured leg.

Figure 19- Case 2: Photograph of wound after 2 weeks of treatment with Manuka honey.
(Courtesy of Professor Doctor Andrew Dart)



Two weeks after wounding, the bandages were removed and this photograph shows a healthy granulation bed formation. After this photograph, the wound was left without bandage and manuka honey application became daily.

Figure 20- Case 2: Photograph of the wound 2 months after treatment with Manuka honey started. (Courtesy of Professor Doctor Andrew Dart)



Two months after wounding, contraction of the wound is noticed as well as epithelialization of the edges of the wound. The horse was maintained in treatment throughout healing and the wound contracted and epithelialization started once contraction stopped and the wound became fully covered. The Quarter-horse went back to western performance.

4.3.Case 3

This is a case of a miniature horse that had a colic episode and flunixin meglumine was administered for the colic signs. The injection went perivascular and one week later the miniature horse presented at the hospital with sloughed neck. Wound went from one side to the other, and the left carotid was lost. The pony was scoped and no laryngeal hemiplegia was observed.

Figure 21 and 22: Photographs of the neck of a 6 year-old miniature horse 10 days after receiving a perivascular injection of flunixin meglumine. (Courtesy of Professor Doctor Andrew Dart)



The horse went under general anesthesia and the wound was debrided, after the surgery, topical Manuka honey UMF 20 was applied twice daily along with a broad-spectrum antimicrobial therapy and non-steroidal anti-inflammatory.

Figure 23: Photograph of the neck wound seven days after the treatment with manuka honey started. (Courtesy of Professor Doctor Andrew Dart)



Seven days after treatment with Manuka honey started the wound had a great progress with healthy granulation tissue forming and covering the wound.

Five weeks after treatment the wound presented completed healed.

Figure 24 and 25: Photographs showing the outcome of the treatment, the scar and the skin completely regenerated in the end of the treatment.(Courtesy of Professor Doctor Andrew Dart)



A study made by Wilmink, Stolk, Weeren, & Barneveld in 2010, concluded that second intention healing of wounds occurred faster in ponies than it does in horses. The same study also showed that wounds on the body healed significantly faster than wounds on the limbs. Second-intention wound healing is different in both cases due to the greater contribution of contraction to the healing process. The inflammatory response appears to be more effective in ponies than in horses, resulting in the more rapid formation of a healthy granulation bed. There is a remarkable delay in the healing of limb wounds in horses.

5. Discussion

This present study has two major objectives: (1) a systematic literature review, based in inclusion and exclusion criteria, assessing the treatment of wounds in Veterinary medicine with Manuka honey in the last decade, in order to analyze the results and see if we can adequate them into clinical practice; (2) the presentation and development of three clinical cases that were observed during my internship and where Manuka honey treatment was applied as part of the therapy.

5.1. Advantages of Manuka honey

Manuka honey presents several components that enhance wound healing. Antioxidant activity, low pH, high sugar content which enables an osmotic activity, deodorizes the wound, has a debridement action by creating a moisturizing wound environment, but more importantly, it has the capacity to modulate the initial inflammatory phases, as well as modifying the wound environment acting as an antimicrobial topical agent.

Another advantage of Manuka is that it is not affected by catalase, an enzyme present in body fluids, as well as not being affected by gamma-irradiation which makes it possible for manuka honey to be sterilized and still maintain full activity.

The fact that Manuka honey can increase activity with storage time, meanwhile other honey types might decrease, is also a good characteristic, and in practice, an important one.

5.2. Manuka honey as an anti-inflammatory agent

Manuka honey enhances wound healing by modulating the initial inflammatory response through by having the 5.8 kDa active component of manuka honey activating the toll-like receptor 4 on monocytes to enhance production of cytokines IL- β , IL-6 and TNF- α important in tissue repair and regeneration that regulate fibroblast production and angiogenesis (Tonks *et al.*, 2007).

This fact was clear, either in the review of literature where all the research done *in vivo* presented a marked anti-inflammatory activity when compared to control groups, as well as in the clinical cases that were presented, with all of them having a great evolution of the bed wound into a healthy tissue.

5.3. Manuka honey as an antimicrobial agent

Manuka honey acts as an antimicrobial mostly because of the presence of an organic compound named methylglyoxal that results from a non-enzymatic conversion of a chemical found in high concentrations in the flower of the manuka tree, dihydroxyacetone. Methylglyoxal acts by creating a combination of enzymatic and non-enzymatic processes that involve the capacity to interact with macromolecules such as DNA and RNA (Tramuta et al., 2017).

In the performed review of literature, several research papers assessed, using different laboratory methods, the antimicrobial activity of manuka honey. The overall results were positive, and manuka honey showed to have a great antimicrobial effect against almost every bacteria that is commonly present in veterinary clinical cases of infections.

Manuka honey acts differently depending on the UMF rating. In the studies assessed in this review, UMF 20 is shown to have the overall best antimicrobial effect when applied in contaminated wounds, this is one of the reasons why as the UMF rate rises, so does the price.

In the clinical cases, UMF 20 was also the chosen treatment, as it is believed to have a greater and faster effect in severe contaminated wounds.

5.4. Action of manuka honey against resistant bacteria

One of the most important characteristics of manuka honey, is the action that it has in biofilms and in resistant bacteria. Exposure of bacteria to manuka honey *in vitro* has demonstrated downregulation of genes coding for surface-binding proteins that are important in biofilm formation. Manuka honey is thought to prevent biofilm maturation when in low concentrations, by disrupting cellular concentrations, while higher concentrations are able to penetrate and detach established biofilms (Westgate, Percival, Knottenbelt, Clegg, & Cochrane, 2011).

Manuka honey when tested against resistant bacteria is the studies that were reviewed, performed the best outcome when compared to other honeys against the resistant bacteria, of specifically importance against MRSA.

5.5. Other types of honey and its capacity as a possible wound healing dressing

Variation in the type and level of antimicrobial activity in honey is associated with the floral source (geographical location, environmental conditions, bee-related factors), so all types of different honeys must be assessed in order to analyze their activity.

Evidence about the performance of other types of honey was assessed (including locally produced honeys and medical honeys). In some species the outcome was satisfying, having sometimes characteristics comparable to the ones of Manuka honey. These studies are usually made in order to reduce the costs of having to buy expensive medical honeys for medical use. This represents the possibility of having cheaper alternatives to manuka honey, while having similar actions. On the other hand, as also assessed in the review made in this study, precautions should be taken when talking about non-sterilized honeys, because it is shown that a wide range of these present bacterial contamination that can eventually harm the patients when applied. So a sterilization method should be considered.

5.6. Short or long-term treatment

While the advantageous effect of application of manuka honey to open wounds on the distal limb of horses is mostly associated with minimizing wound retraction, proceeding daily application of manuka honey gel beyond 12 days after wound creation appears to contribute to an amelioration in overall wound healing. Although not statistically significant, this effect is shown to be associated with a faster rate of healing between day 7 and 21, after that time, there appeared to be no benefit in continuing to apply manuka honey gel. The study addressed in the review, is a study that assesses this situation in surgically created wounds. Clinically, wounds are often larger and associated with substantial tissue trauma so the results should be extrapolated with caution when using manuka honey in a clinical setting. Nevertheless, the results suggest that manuka honey is most effective as a topical treatment of distal limb wounds in horses in the first 3 weeks after wounding. The later phases of second intention healing may be supported more by alternative treatments (Bischofberger *et al.*, 2013).

5.7. Bandaged or unbandaged wounds

Prolonged bandaging of wounds has been shown to gradually induce dysregulated healing and lastly the production of the excessive granulation tissue that is frequently related with naturally occurring distal limb wounds. Semi-occlusive bandages can reduce gas exchange between the granulation bed and the surrounding environment, exacerbating local hypoxia and promoting excess granulation tissue production in equine lower limb wounds. Recent studies investigating the effects of Manuka honey on distal limb wounds have applied a semi-occlusive bandage for approximately 12 days to try and to create a wound environment that better replicates the environment in naturally occurring wounds (Bischofberger et al., 2011). If the bandage is removed approximately 12 days after wound creation, wounds generally do not progress to developing excessive granulation tissue; however, the granulation bed does not appear as organized as that of comparable unbandaged wounds. Although this model has been used, the histological effects of applying a bandage to the wound for 12 days have not been evaluated (Bischofberger *et al.*, 2016).

5.8. Benefits of conducting a literature review

There are benefits in conducting a literature review using inclusion and exclusion criteria, not only for the person that conducts the review, but also for the public reading it.

Literature reviews are useful in providing an overview of the existing evidence on the topic. They identified the key questions and the need of further research. An overall assessment of the quality of the research that exists on the topic is made as well as an evaluation on how the methods and results are described in the reviews and if they are clear and complete containing all the necessary information. The results of a literature review make a valuable tool for the person reading it, as it gives the overall conclusions of several studies with similar designs, making it easier to have an opinion about a subject, by summing it up. The risk of bias is assessed and so it ensures that all the studies reviewed are relevant and reliable.

5.9. Benefits of clinical cases presentation

The aim with the presentation of the three clinical cases, was not only to show what I have seen during my internship, but as well to validate with a personal statement the action Manuka honey has in wound healing with real cases where a great outcome was witnessed.

The studies being performed with Manuka honey don't entirely recreate the action that honey effectively has, due to the recreation of natural occurring wounds not being done, so this aims to complement other approaches. These wounds show a great level of wound retraction and contamination as well as severe tissue avulsion, making it clear that second intention healing is the only way to go.

5.10. Limitations

The main limitation with clinical cases is that there are no control groups, as well as no possibility of establishing cause-effect relationship.

Histological evidence was not recorded in these cases, so the follow-up evaluation was mainly by macroscopically assessment of the wounds, which can endanger of over-interpretation of the results.

Complementary therapy was performed in both cases with broad-spectrum antimicrobials and non-steroidal anti-inflammatory treatment, so the results obtained may not be entirely due to the topical application of Manuka honey.

6. Conclusion

Wounds in horses are one of the most common reasons why an Equine Veterinarian is called, and also a common reason to get the horse out of competing for some time. These wounds are usually characterized by having a severe tissue avulsion, great contamination, and a weak inflammatory response. When inflammation fails to resolve, and a chronic inflammatory response is established, the process can become dysregulated, resulting in pathologic wound repair and the accumulation of permanent fibrotic scar tissue at the site of injury. This fibrosis is characterized by the excessive accumulation of ECM components, including collagens, fibronectin, and hyaluronic acid at the site of injury, leading to a decrease in organ function and, in some cases, organ failure and death (Stashak & Theoret, 2008).

Increasingly, bacterial resistance is becoming a worldwide problem. Alternative and natural treatments are being investigated, to try to fight this situation. Manuka honey is the most well studied honey being used for this purpose at the present time.

In this study a review of literature aimed to demonstrate if there is evidence in Veterinary Medicine that honey is useful as a wound dressing to enhance healing was performed. To complete this study, three cases of severe wounds in horses were assessed in this study with the aim of showing that a treatment with Manuka honey can benefit wound healing.

This study concludes that Manuka honey represents an innovation treatment in the area of equine wound healing. As a dressing it has the potential to improve the healing of wounds and shows a promising outcome as an antimicrobial agent, especially against resistant bacteria.

Conclusions can be made on how effective Manuka honey can be when a mixed with water-based gel thereby decreasing the costs of the treatment. Further Manuka honey action enhances wound healing by acting as an immunomodulator and anti-inflammatory, specifically in the first phases of wound healing (Bischofberger *et al.*, 2016).

Furthermore, as new information is arising on the bioactivity of different honey varieties, it may be possible to match the features of different varieties of honey to treatment of different stages of the wound healing process, and by that decreasing the costs of treating wounds in horses, that are also a problem for the owners, also due to the length that these treatments require. Treatment with Manuka honey can become expensive, especially if a high UMF is required, and some studies have shown that there are honeys that match the same characteristics as manuka honey, and some have an even better outcome when working as an antimicrobial, so clinical application of these studies is warranted to compare the outcomes.

7. Future directions

Wounds are very common in horses, especially traumatic lacerations of the distal limb, and the treatment of these wounds can be very expensive. With the rising costs of treating problematic complex wounds in both human and veterinary medicine, it would be an advantage to find ways to optimize the effectiveness of the treatment.

There are still a lot of components where further investigation is warranted when it comes to the exact knowledge on how Manuka honey acts, either as an immunomodulator but also as an antimicrobial agent.

Studies should be assessed in order to evaluate further dilutions of manuka honey to determine what concentration is needed to retain the therapeutic effects and efficacy, and at what dilution the activity is lost. This would bring economy benefits.

Currently, limited research using wound healing models suggest that the effects of Manuka honey on the initial inflammatory response plays an important role in enhancing the early stages of second intention healing of distal limb wounds in horses.

A way to try to replicate a naturally occurring wound, when using wound healing models in research, would be beneficial, in order to obtain results that are closer to the reality.

In terms of Manuka honey, there is still the need to improve the therapeutic approach. It would be beneficial to understand if a Manuka honey with UMF 20 is needed to achieve the benefits of treatment in all wounds or only in the wounds that present a great degree of contamination or are contaminated with antibiotic resistance organisms. Further studies on what is the exact activity that generic honeys perform in wounds that are not contaminated are also warranted.

The development of a variety of dressings specific for wound types would improve the outcome and treatment could be established in a more cost-effective way.

Further research should be targeted at determining whether the honey derived from other species of plants have medicinal properties and can be used topically to enhance wound healing. Studies on the possible synergy between Manuka honey and antibiotics should be transferred into a clinical trial, so that honey could become a potential product to be applied parenterally or into joints or synovial structures.

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