THE INFLUENCE OF NEGATIVE PRESSURE WOUND THERAPY ON SECOND INTENTION WOUND HEALING IN THE EQUINE DISTAL LIMB

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LIST OF ABBREVIATIONS

AgPU	Silver impregnated polyurethane ether foam
ANOVA	Analysis of variance
CFU	Colony-forming units
CNA	Columbia Colistin and Nalidixic acid agar
DP	Digital photoplanimetry
DPB	Digital photoplanimetry-based
ECM	Extracellular matrix
EPS	Extracellular polymeric substance
EpSCs	Epithelial-like stem/progenitors cells
G	Gauge
HAIS	Histological acute inflammation score
HE	Hematoxylin and eosin
HRS	Histological repair score
ICC	intra-class correlation test
IM	Intramuscularly
IV	Intravenously
LB	Laser beam
MANOVA	Multivariate analysis of variance
MRSA	Methicillin resistant *Staphylococcus aureus*
MSCs	Mesenchymal stem cells
NDT	Negatieve druktherapie
NPWT	Negative pressure wound therapy
NPWT-AgPU	NPWT using a silver impregnated polyurethane foam

NPWT-PU	NPWT using a normal polyurethane foam
NPWT-PVA	NPWT using a polyvinyl alcohol foam
NSAIDs	Nonsteroidal anti-inflammatory drugs
PAS	Periodic acid schiff
PBS	Phosphate buffered saline
PHMB	Polyhexamethylene biguanide
PRP	Platelet rich plasma
PSI	Pounds per square inch
PO	Per os
PU	Polyurethane ether foam
PVA	Polyvinyl alcohol foam
R	Reference
RB	Relative bias
REF	Reference
SC	Subcutaneously
SD	Standard deviation
SEM	Standard error of the measurement
α-SMA	Alpha smooth muscle actin
TGF-β1	Transforming growth factor β1
T	Time point
TGF-β3	Transforming growth factor β3
V.A.C.	Vacuum Assisted Closure

“*The important thing is never to stop questioning*”

Albert Einstein

GENERAL INTRODUCTION

THE QUIRKS OF EQUINE WOUND HEALING

1 INTRODUCTION

The body of both humans and horses reacts to a tissue injury by initiating a self-repair system. If this injury is situated in the skin the body starts up a wound healing response. The body wants to prevent further damage and mend the tissue defect (Diegelmann and Evans, 2004; Theoret, 2008a; Provost, 2012). Wound healing is mainly accomplished by repair instead of regeneration. The missing tissue is not replaced by an exact copy, but the defect is filled with undifferentiated scar tissue (Theoret, 2008a). Horses in particular are prone to traumatic wounds (Theoret et al., 2016). These animals are plain feeders with a low territorial defence drift. They rely on caution, speed and agility for their survival. In case of danger, horses use their enormous muscle mass to achieve top speeds to put as much distance as possible between them and the potential threat (McGreevy, 2004). This flight instinct causes horses to regularly sustain traumatic wounds. Throughout this PhD study, focus will be set on the wound healing mechanisms in horses, the specific problems encountered in this field and possible solutions. Since a lot of information on wound healing in horses is extrapolated from human medicine, occasionally a link will be made between equine and human medicine (Theoret, 2008a; Provost, 2012).

The four macroscopically visible stages of wound healing

The healing of traumatic wounds and wounds in general can be divided into four different macroscopically visible stages: acute inflammation, formation of granulation tissue, wound contraction and epithelialization (Fig. 1) (Wilmink, 2008).

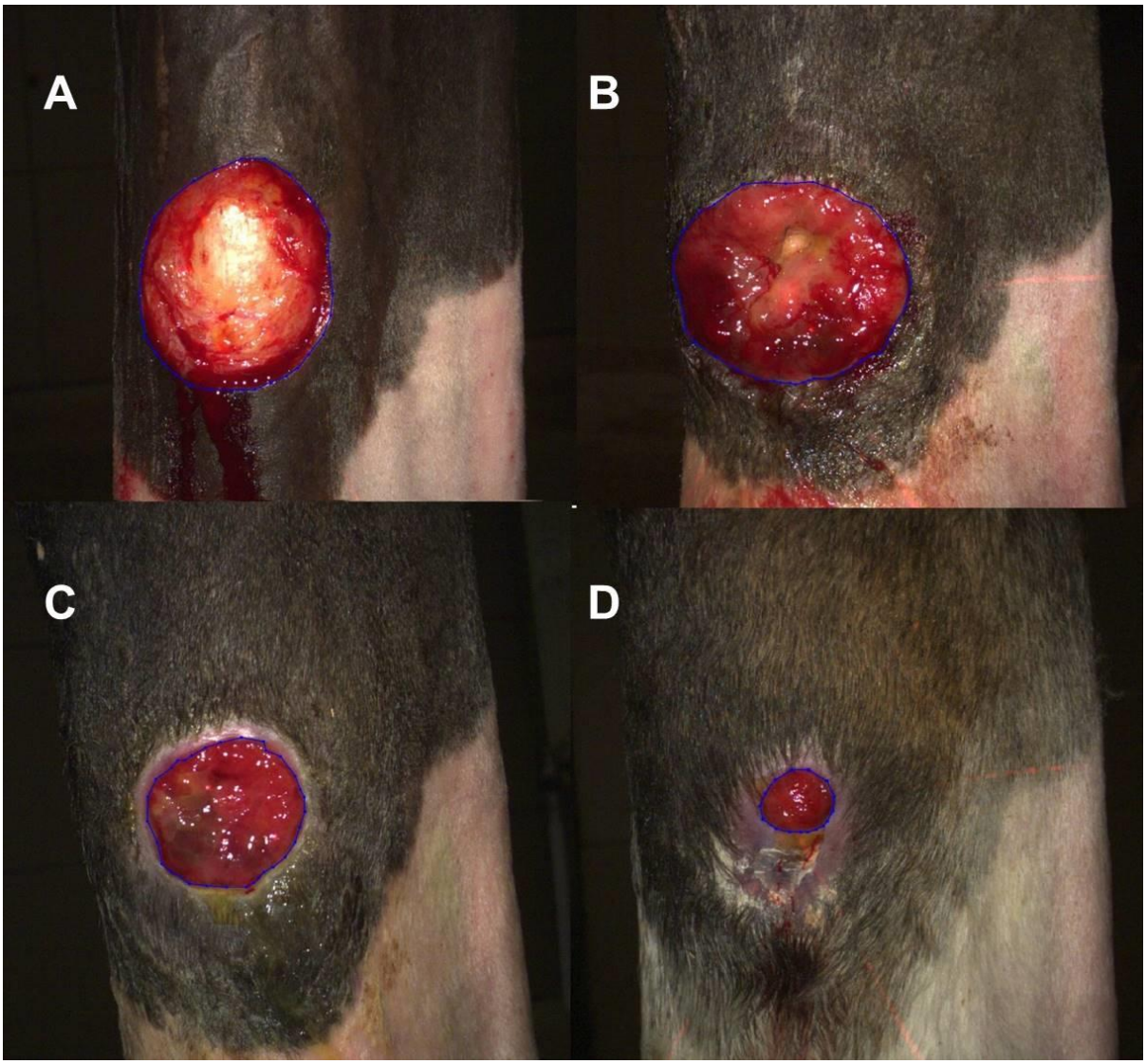


Figure 1. An overview of the 4 macroscopically visible stages of wound healing in an experimental wound.

The wound boundaries are indicated by the blue line. (A) Acute inflammation of the wound with a hyperaemic appearance and oedematous swelling of the subcutaneous tissue. (B) granulation tissue formation. (C) Wound contraction. (D) Epithelialization.

During the acute inflammation, polymorphonuclear cells such as neutrophils migrate to the wound bed within a matter of minutes after skin disruption. These cells are the first line defence of the body by removing debris and bacteria through phagocytosis. Soon after the influx of neutrophils, the number of macrophages rapidly increases to assist in the debridement of the wound. In contrast to the neutrophils, macrophages play a crucial role in the induction and advancement of the granulation tissue formation (Leibovich and Ross, 1975; Theoret, 2008a).

The stage following and partially overlapping the acute inflammation, is the formation of granulation tissue. During this stage the inflammation dwindles and fibroblasts and endothelial cells massively appear into the wound under stimulation of the cytokines produced by the local macrophages. The fibroblasts migrate along the fibrin blood clot and produce extracellular matrix (ECM) to fill up the wound, while the endothelial cells form new capillary buds to transport oxygen and nutrients to the wound bed necessary for the cell proliferation and metabolism. The combination of the freshly formed capillaries and the ECM with the fibroblasts forms a bed of granulation tissue with a typical pink to red appearance because of the elevated presence of small blood vessels. This base of granulation tissue is needed for the wound to proceed to the next stages of wound healing namely the wound contraction and epithelialization (Singer and Clark, 1999).

In order for the wound to contract, part of the fibroblasts within the wound bed differentiate into a specialized phenotype rich of an intra-cellular alpha smooth muscle actin (α-SMA) microfilamentous system, the so-called myofibroblasts. These myofibroblasts are connected to the ECM and to each other, so when their intra-cellular microfilamentous system contracts they literally draw the wound edges together to the centre of the wound (Clark, 1993; Singer and Clark, 1999) (Fig. 2). This stage is very important in determining the speed and the final cosmetic outcome of the wound healing, since the next stage of formation of new epithelium is a slow process which can easily be damaged (Theoret, 2008a; Wilmink, 2008).

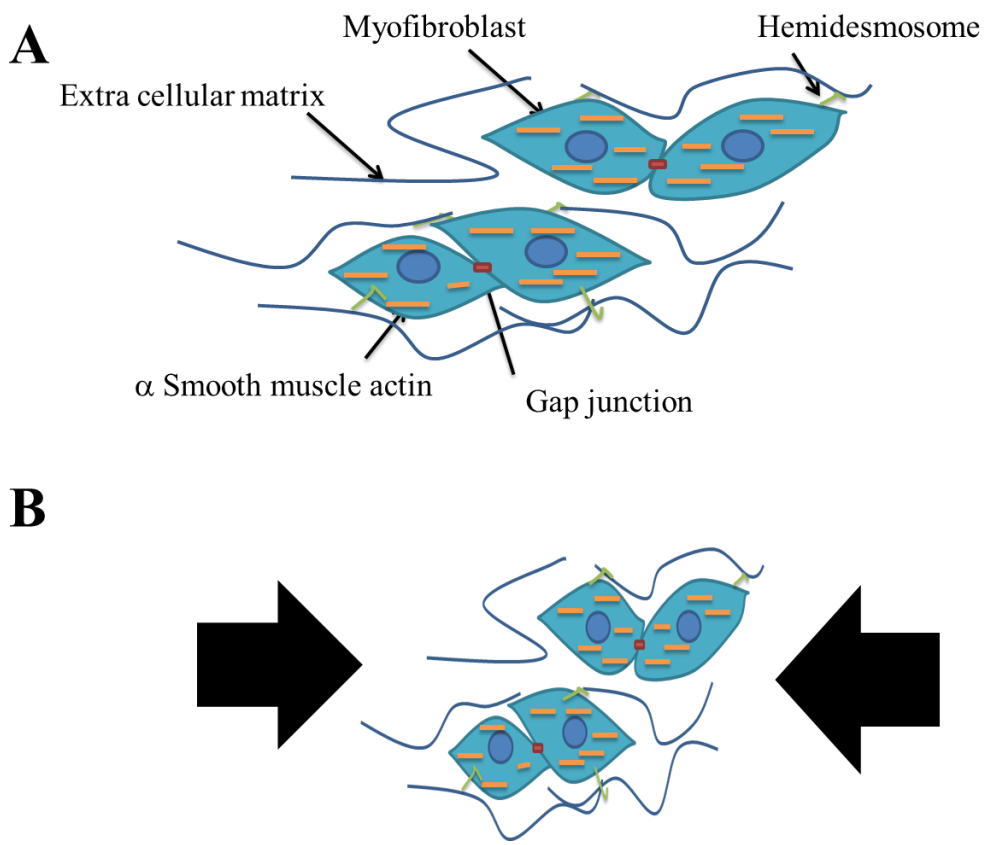


Figure 2. A schematic presentation of the wound contraction on microscopic level. (A) the myofibroblasts are a differentiated phenotype of fibroblasts, which are rich of an intra-cellular alpha smooth muscle actin microfilamentous system. The myofibroblasts lay within the wound bed and are connected to each other with gap junctions and to the extra cellular matrix in their environment with hemidesmosomes. (B) When the intra-cellular microfilamentous system contracts the myofibroblasts are drawn to each other and they pull on the extracellular matrix, so the wound edges are drawn together to the centre of the wound.

The last macroscopically visible stage of wound healing is the epithelialization. In this stage the epithelial cells migrate from the intact wound edges over the granulation bed to cover the wound bed with a new thin layer of epithelium. The new epithelium is macroscopically visible as a pink rim along the wound edge (Fig.1d). This process is slow (about 0.1 mm per day in distal limb wounds in horses) and the new layer of epithelium is very brittle because it is not yet attached to the underlying epidermis. The new epithelium also lacks skin adnexa such as hair follicles, sweat and sebaceous glands, which makes its dry and less supple. The part of the wound that heals by epithelialization will always be visible as a superficial scar (Jacobs, et al., 1984; Wilmink, 2008).

Wound remodelling

Wound healing is a slow process. In wound healing by second intention (the wound is left open to heal), the fastest rate of granulation tissue formation occurs between 7 to 14 days after injury. This is also the period with fastest increase in tensile strength of the wound. However, at this time point less than 20 % of the final wound strength is achieved (Singer and Clark, 1999). During wound remodelling, the granulation tissue is slowly converted into an avascular and rather acellular scar and the scar tissue gets more organized. This process continues for up to 2 years. In first intention healing wounds, the final scar reaches about the same tensile strength as the original skin at 6 weeks (Chism et al., 2000). However, in second intention healing wounds, scars are weaker, less extensible, and less tough than normal skin (Corr et al., 2009). Therefore, modern wound treatments should focus on speeding up the different stages or overall process of wound healing and maximizing the quality of the final scar tissue.

2 THE ABERRANT WOUND HEALING IN HORSES

Generally, a wound can be closed or left open to heal. When a wound is closed, the wound edges are opposed together and the wound heals without granulation tissue formation. This is called wound healing by first or primary intention (Wilmink et al., 2002). In contrast, When the wound is left open to heal, granulation tissue is formed. This process is called wound healing by second intention. Wound healing by first intention accelerates the wound healing process and gives a better cosmetic result than wound healing by second intention. However wound healing by first intention is often not feasible in horses because of a high skin tension, massive tissue loss, pronounced contamination or a long duration from onset of the injury (Theoret, 2008b). Moreover, second intention wound healing can also occur when a primary closed wound dehisces because of for example infection, movement at the wound site or high tension on the wound margins (Wilmink et al., 2002).

First intention wound healing

As already mentioned, wound healing by first intention is always preferred because it is faster and gives better cosmetic results compared to second intention wound healing (Wilmink et al., 2002). However, Wilmink et al. (2002) demonstrated that first intention wound healing is more successful in ponies (wither height < 1.48 m) than in horses (41% vs 26% respectively). Ponies also have less tendency to form a sequester (a necrotic piece of bone) in case the cortical bone is affected during trauma (4% vs 31 % in horses). Although the ponies in the study of Wilmink et al. (2002) generally had a less favourable prognosis because their wounds were deeper, less thoroughly debrided and the ponies received less antibiotics, the wounds of the horses dehisced more, probably due to a higher presence of wound infection. Wilmink et al. (2002) suggested that horses had a less efficient inflammatory response than ponies, due to either genetics or the higher use of nonsteroidal anti-inflammatory drugs (NSAIDs) in horses, which led to a less effective reduction of the bacterial contamination in the horse wounds.

Second intention wound healing

Wounds in horses are often left open to heal by second intention because of massive tissue loss, a high skin tension and excessive bacterial contamination (Theoret, 2008b). To investigate the difference in second intention wound healing between ponies and horses, Wilmink et al. (1999a, 1999b) performed a study which provided insight in the basic biology of second intention wound healing within the equine species. In their study on the macroscopic aspects of wound healing Wilmink et al. (1999a) found that ponies healed significantly faster than horses, and body wounds healed faster than distal limb wounds. An

important contributor to this faster wound healing was the greater amount of wound contraction in ponies compared to horses and in body wounds compared to distal limb wounds. Moreover, the distal limb wounds of horses retracted for a longer period of time and the surface area almost doubled compared to the beginning of the study. Because the distal limb wounds had less wound contraction, these wounds had to heal mainly by epithelialization, which resulted in a larger and more friable scar compared to pony wounds and body wounds.

In the study on the histological aspects Wilmink et al. (1999b) reported that ponies had an earlier and stronger inflammatory response, while in horses the onset of the inflammatory response was slower and persisted over time. According to Wilmink et al. (1999b), the stronger inflammatory response in ponies led to a more efficient and faster wound debridement with as a consequence formation of healthier granulation tissue. The swift and efficient inflammatory response in ponies would also be more successful to prevent infection (Wilmink et al., 2002). In contrast, granulation tissue formation was faster in horse wounds, but evolved more frequently to exuberant granulation tissue, especially in distal limb wounds. Wilmink et al. (1999b) also found that fibroblasts kept proliferating in horse wounds and microscopically the granulation tissue was more chaotic.

Conclusion

Horses have an inefficient inflammatory response, which often leads to chronic inflammation, especially in distal limb wounds (distally of the hock and carpal joint). This chronic inflammation leads to a low amount of wound contraction, which is already limited by the high skin tension on the horses' distal limbs, and to the formation of low quality granulation tissue. Moreover, the slacking inflammatory response causes a less efficient wound debridement, which increases the chance of wound infection (Wilmink, 2008). Wound healing is even further impaired at distal limb wounds because of a decreased oxygenation and more occlusion of microvessels, which contribute to the chronic inflammation and exuberant granulation tissue formation (Lepault et al., 2005; Celeste et al., 2011). Therefore, horses have an aberrant wound healing especially at their distal limbs and an ideal wound treatment should address these issues to improve healing.

3 SIMILARITIES BETWEEN WOUND HEALING IN HORSES AND HUMANS

Both human and veterinary medicine have the goal to find an appropriate treatment for each wound or even in the future discover a way to achieve wound regeneration instead of only repair. Therefore, exchange of knowledge on wound treatments and wound healing mechanisms benefits both fields.

Similarities between wound healing in horses and humans

Research performed on wound healing in horses can potentially be extrapolated to human medicine (Theoret and Wilmink, 2013; Wilmink, 2014). Second intention wound healing at the distal limbs of horses resembles to a certain extent healing of lower leg and feet wounds in humans. Both types of wounds heal mainly by epithelialization (± 70%) and less by contraction (± 30%), so in both cases a superficial scar remains visible after healing which is often friable and easily traumatized (Theoret and Wilmink, 2013). Moreover, distal limb wounds in horses display an abnormal wound healing, which is reminiscent to chronic non-healing wounds in humans such as pressure ulcers, diabetic foot ulcers or venous leg ulcers (Diegelmann and Evans; 2004; Theoret and Wilmink, 2013; Baltzis et al., 2014; Wilmink, 2014). In horses, distal limb wounds have a slow starting inflammatory response, which is initially weak and persists over time leading to chronic inflammation (Wilmink et al. 1999b). In humans, chronic wounds also display a chronic inflammation, which prevents the wound to heal in a timely fashion (Diegelmann and Evans, 2004). In both cases, the chronic inflammation has to be resolved in order for the wound to progress.

Biofilms are frequently present in chronic non-healing wounds in humans (James et al., 2008). Similarly, in horses, the chronic inflammation combined with heavily contaminated housing circumstances often results in biofilm formation in distal limb wounds (Westgate et al., 2011; Wilmink, 2014).

Another reason why horses are interesting to use as model for human wound healing is their genetic predisposition to develop exuberant granulation tissue and hypertrophic scars (Wilmink, 2014). Exuberant granulation tissue resembles keloids in humans, because they both display a fibroproliferative overgrowth expanding upwards and outside the original borders of the wound. However, exuberant granulation tissue is not epithelialized (Theoret et al., 2013). Hypertrophic scars in horses are very much the same as in humans and differ from keloids as they are both raised, but hypertrophic scars do not expand beyond the original wound margins (Theoret et al., 2013). Moreover, next to an inherent chronic inflammation and a tendency to develop fibroproliferative disorders, horses have a decreased blood flow and oxygenation in distal limb wounds (Lepault et al, 2005; Celeste et

al., 2011). This complication is also seen in wounds of human diabetic patients because of micro- and macrovascular disease.

The horses as a human wound model: considerations

Although horses are potentially interesting to use as a wound model for humans, certain limitations of their use as experimental animals have to be borne in mind. Horses are large animals and are more difficult and expensive to house than the standard rodent experimental animals. Trained staff is also needed to correctly and safely handle these animals (Theoret and Wilmink, 2013). Moreover, species-specific diagnostic laboratory tools for horses are still scarce (Theoret and Wilmink, 2013). These limitations have to be considered before commencing an *in vivo* experiment using horses, so that the information gained from each experiment is worth the investment.

STANDARD OF CARE FOR EQUINE SECOND INTENTION HEALING WOUNDS

1 INTRODUCTION

Every equine practitioner confronted with a traumatic wound in a horse ideally wants to provide the best possible treatment for the animal, while minimizing the costs for the owner. In the best-case scenario, the wound can be closed and left to heal by first intention. This technique provides a superior esthetical and functional outcome over second intention healing (Stashak, 2008a). However, wounds in horses often dehisce after primary closure or are immediately left open to heal by second intention because of massive tissue loss, excessive bacterial contamination, a long duration since the initial trauma or high skin tension (Wilmink et al., 2002; Theoret, 2008b). There are many options to treat an open wound and currently there is no consensus or standard protocol, partly because of the large variation in types of open wounds. Therefore, a guideline for the treatment of a second intention healing wound is presented in this chapter based on the current literature and the standard procedures used in our institution. This guideline will entail general recommendations, but every wound should be evaluated individually and treated according to its specific characteristics.

2 WOUND PREPARATION AND EVALUATION

Hair removal

To efficiently evaluate the extent of a wound and to reduce bacterial contamination, the hairs on the skin surrounding the wound should be removed (Stashak, 2008b). Sometimes it is necessary to groom the hairs first because of macroscopically visible dirt. Before removing the dirt or the hairs, the wound should be protected with sterile moistened gauzes or a sterile water-based lubricant such as KY-jelly (Johnson & Johnson), so the dirt and/or hairs do not fall into the wound (Stashak, 2008b). The hairs surrounding the wound should be slightly dampened to further decrease the risk of hairs falling into the wound (Stashak, 2008b). To remove the hairs an electric clipper is preferred over a razor blade, since shaving with a razor blade often gives additional skin injury (Jose and Dignon, 2013). Another option is to use a depilatory cream, but from personal experience we noticed that horses often have a skin reaction to this type of creams. Moreover, an electric clipper is a much faster method to remove the hairs compared to a depilatory cream, which has to remain in contact with the hairs for 5 to 20 minutes. After removal of the hairs, the skin should be scrubbed with an antiseptic soap such as chlorhexidine digluconate detergent solution (Hibiscrub, Mölnlycke health care) to prevent contamination (for example shaved hairs) to be dragged into the wound during exploration.

Wound irrigation and cleansing

Wound irrigation and cleansing is an essential step to evaluate and treat the wound. The main goal of wound irrigation and cleansing is to decrease the amount of contamination and to remove crusts and slough (Fig. 3). However, with cleansing/irrigation only superficial contamination and loosely adhered tissue remnants and micro-organisms are removed (Stashak, 2008b). For a more extensive cleaning of the wound, a thorough debridement is necessary. Wound cleansing can be performed using various methods and antiseptics (Stashak, 2008b). In this guideline, only the most frequently used techniques in our institution will be described.

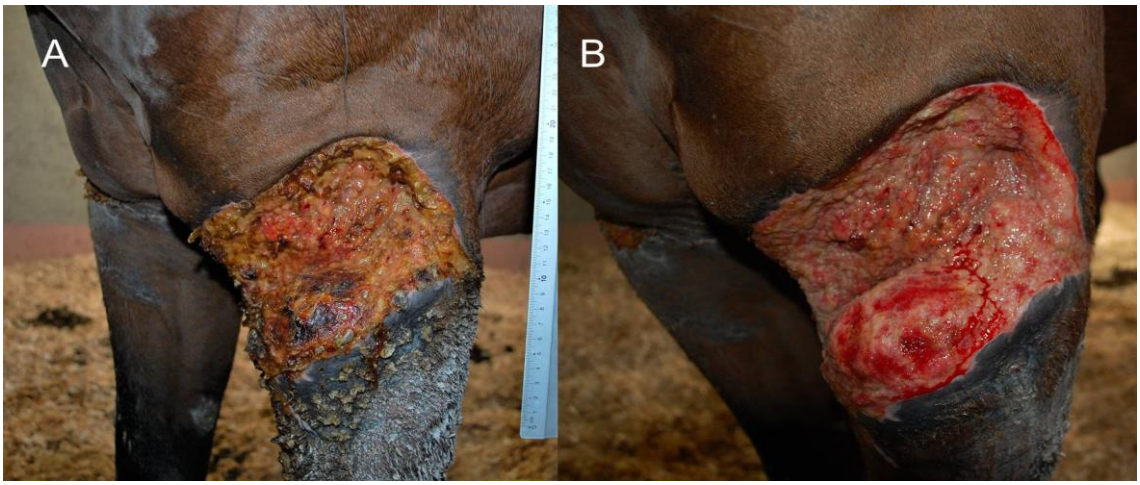


Figure 3. A second intention healing wound before (A) and after (B) cleansing. (A) Before wound cleansing, the wound surface is covered with a yellow slough/necrotic tissue and crusts. (B) After wound cleansing, the actual colour of the granulation tissue is visible and the extent of the wound can be assessed more easily. All superficial contamination has been removed.

Wound irrigation is performed to dislodge bacteria, loosely adhered debris and devitalized tissue. Generally, a pressure from 7 to 15 pounds per square inch (PSI) is recommended (Stashak, 2008b). In the institution of the author, two methods are generally used to flush the wound: (1) either a 35 mL syringe and a 19 gauge (G) needle (of which the metal part is broken off) is used delivering about 8 PSI to the wound surface, or (2), more frequently, puncture holes are made with a 16 G needle in the top of a plastic bottle of sterile saline solution (0.9% NaCl). The bottle is squeezed to deliver a stream of solution to the wound surface (Fig. 4). The pressure generated with the latter technique is lower than the pressure generated with the 35 mL syringe (Pint et al. 2011).

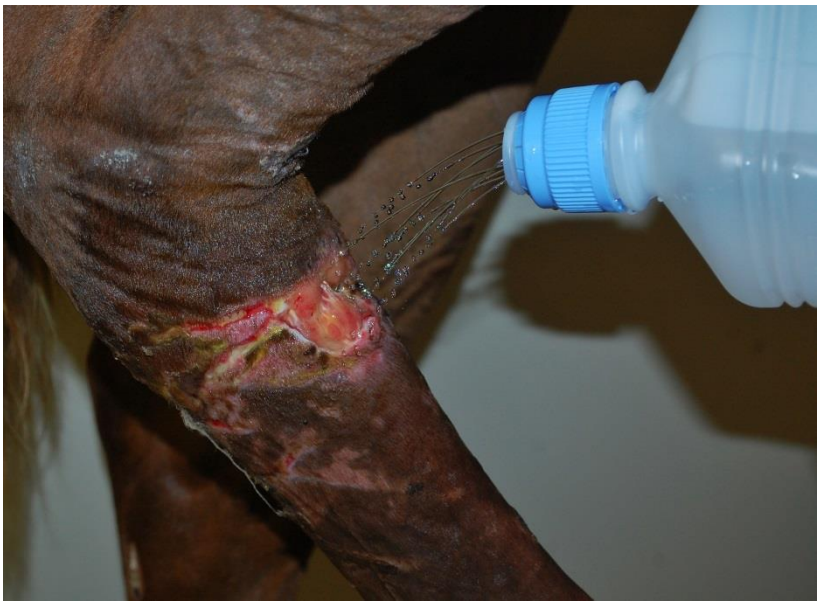


Figure 4. Irrigation of a second intention healing wound. The wound is located at the right metatarsus and is irrigated using a plastic bottle filled with sterile saline solution (0.9% NaCl) of which the top has been punctured several times with a 16 G needle.

The choice of irrigation solution is dependent upon the clinical assessment of the wound. If the wound is clean and the granulation tissue is nicely smooth and pink, a sterile saline solution (0.9% NaCl) is used (Fig. 5a) (Stashak, 2008b). However, if the wound shows any signs of infection (table 1) (Fig. 4b), an antiseptic needs to be added to the sterile saline solution. Commonly chlorhexidine digluconate is added to obtain a 0.05% solution. Povidone-iodine is a less suitable antiseptic to flush a wound, since it is rapidly deactivated by the presence of organic tissue such as blood, pus and fat (Misra, 2003). This can explain the variable results of the anti-bacterial effect of povidone-iodine in *in vivo* studies (Burks, 1998). Moreover, povidone-iodine makes is it more difficult to assess the wound because it stains the tissue (Misra, 2003).

Table 1. An overview of possible signs of infection in a second intention healing wound.

- Exudate (oozed through the third layer of the bandage) clear / sanguineous / purulent
- Red granulation tissue (beefy aspect)
- Yellow necrosis/slough/discoloration of the granulation tissue
- Black necrosis/slough/discoloration of the granulation tissue
- Exuberant granulation tissue
- Oedematous granulation tissue (glassy, shiny aspect)
- Friable granulation tissue (easily bleeding when probing the surface and base of the wound)
- Oedema around the wound/ part of the limb /entire limb
- Unpleasant odour
- Pain (it is difficult to touch the wound even when the horse is sedated)

Figure 5. Two second intention healing wounds.
The granulation tissue in the left wound (A) is nice and smooth and has a healthy pink colour. In contrast, the wound on the right (B) looks highly inflamed and infected. The granulation tissue is irregular, friable and has a dark red to black colour on certain parts of the wound.

Wound cleansing is performed by rubbing a sterile soaked gauze over the wound surface. This technique removes superficial debris and micro-organisms. The solution to soak the gauzes is chosen following the same criteria as described for the wound irrigation solution.

Wound evaluation

After the wound is cleansed and free of visible contamination (Fig. 3), the wound can be re-evaluated for signs for infection (table 1) and explored to assess its extent. It is important to evaluate whether bone or foreign bodies are present, and if there are any communications with synovial structures (Stashak, 2008b). The wound can be explored digitally wearing a sterile non-powdered glove or a sterile probe (Stashak, 2008b). Medical imaging methods (e.g. radiography to visualize bone or ultrasound to visualise soft tissues) can be used to further assess any concomitant complications (Stashak, 2008b). Throughout the rest of this guideline, the treatment of a second intention healing wound that does not show any additional complications such as bone fractures or communications with synovial structures, will be described.

3 WOUND DEBRIDEMENT

Debridement means removal of debris, so in case of a wound this means removal of all nonviable tissue, foreign bodies and micro-organisms to reduce contamination to a minimum. Sometimes wound irrigation and cleansing are sufficient, but if debris is still present after these steps a more thorough wound debridement is necessary. Wound debridement can be performed using different techniques (Stashak, 2008b), but the most practical and cheap method is a sharp debridement using a scalpel blade or curette (Pint et al., 2011). With this technique, all the debris in the superficial layers of the wound is removed moving from one wound edge to the other. This procedure is repeated until viable tissue is reached and the contamination is considered to be minimal. Exposed bone should also be debrided using a curette or some type of rasp. As a general rule, the following motto can be applied '*If in doubt cut it out, if it is skin leave it in*' (Andreas Schwarzkopf). Another important indication for wound debridement is the presence of exuberant granulation tissue, which is a typical problem in distal limb wounds in horses. Granulation tissue is exuberant if the tissue bulges over the adjacent skin or epithelial rim (about 2 à 3 mm) (Wilmink et al., 1999a; Theoret and Wilmink, 2008). This prevents the wound from contracting efficiently and hinders further epithelialization (Wilmink, 2008). Therefore, exuberant granulation tissue has to be trimmed to the level of the adjacent skin in order for the wound to progress. Next to sharp excision the exuberant granulation tissue can also be treated with corticosteroids. Corticosteroids should be applied at the first sign of exuberant granulation tissue formation and should be limited to one or two applications. If corticosteroids are applied for a prolonged time they exhibit negative effects on angiogenesis, wound contraction, and epithelialization. However, corticosteroids are not efficient for treating advanced en very large masses of exuberant granulation tissue. Caustic agents (e.g. silver nitrate) or cryogenic surgery could also be used to treat exuberant granulation tissue, but are not recommended because they induce pronounced necrosis of the granulation tissue and epithelium border, which delays wound healing. Therefore, surgical excision of exuberant granulation tissue is the preferred choice of treatment (Wilmink and van Weeren, 2005).

4 TOPICAL WOUND TREATMENT

There is a plethora of topical wound treatments, some more effective, practical or cheap than others. This guideline will be limited to the most frequently used treatments in our institution. These treatments were chosen based on evidence based medicine, or, when studies were lacking, personal experience and practical considerations. An overview of the topical treatments will be given according to their use in the different phases of wound healing. Because of the increasing problem of antimicrobial resistance of micro-organisms, local antibiotic therapies are discouraged and will not be discussed (Fletcher, 2015). A proper wound debridement and appropriate topical wound treatment using antibacterial dressings are a more recommended alternative over local antibiotics.

Inflammatory phase

The first objective in the inflammatory phase is to minimize contamination and stimulate the inflammatory response, so the immune response can overcome the dwelling bacteria and remove all nonviable tissue. To help cleanse the wound, dressings can be used which have an antibacterial effect and/or which stimulate wound debridement.

Gauze dressings (e.g. Curity™, Kendall; Steripad™, Zeno Phar; …) are an old and cheap method to achieve wound debridement (Stashak 2008c). This type of dressing is absorbent and adherent. The gauze is applied dry to an exudative wound or moistened (with sterile saline solution 0.9% NaCl ± an antiseptic) to a dry wound or if the exudate is very viscous. The gauze is normally left in place for one day. During this period, the gauze dries and gets stuck to the wound bed because fibrin from the wound adheres to the gauze (Stashak, 2008c). When the gauze is then subsequently removed, the superficial layer of the wound bed is removed along with the dressing. This can be rather painful for the horse, so sometimes it is helpful to moisten the dressing before removal. The application of these dressings should be discontinued if a healthy granulation bed is achieved. The non-selective debridement otherwise impedes further wound healing instead of stimulating it because it is too aggressive.

Gauze-like dressings (e.g. Zorbopad™, Millpledge; Melolin™, Smith & Nephew…) are also absorbent but not adherent. They do not have the same debridement effect as a normal gauze dressing and also dehydrate the wound bed because of their absorbent properties (Pint et al., 2011). Therefore, these dressings are less suitable for a second intention healing wound, but can be used for wounds healing by first intention.

There are also antimicrobial gauze dressings available (Kerlix AMD™, Kendall), which have the same mechanism of action as a standard gauze dressing, but are impregnated with polyhexamethylene biguanide (PHMB). These dressings have a broad antimicrobial spectrum (Lee et al., 2004) and thus not only debride the wound but effectively kill bacteria. Again, these dressings are applied dry to a wet wound or wet to a dry wound and can be left in place for two or three days. The roll form of this dressing is also suitable to pack cavities of a wound (Stashak, 2008c). This dressing is routinely used by the authors to treat heavily contaminated second intention healing wounds and is preferred over the standard gauze dressing because of its antimicrobial activity (Fig. 6).

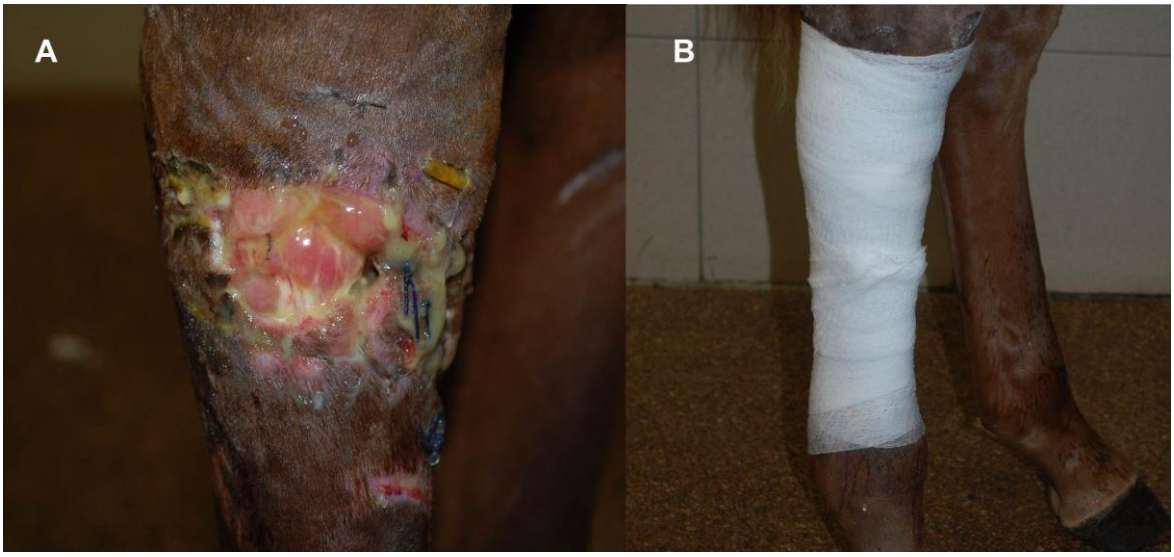


Figure 6. (A) A second intention healing wound with signs of infection. (B) To reduce the bacterial contamination and debride the wound, it is dressed with an antimicrobial gauze dressing (roll form).

Another option to debride a wound using a dressing, is a hypertonic saline dressing (Curity Sodium Chloride dressing, Covidien). This is a gauze dressing impregnated with a 20% NaCl solution. This dressing has a strong osmotic effect on the wound bed, desiccating the bacteria and nonviable tissue and alleviating tissue oedema (Stashak, 2008c). However, this effect is non-selective and thus very aggressive on the overall healing process. This dressing should therefore be limited to three to five applications and the dressing has to be changed daily.

A poultice pad (Animalintex™, Robinson animal health care) is a non-adherent highly absorbent cotton pad dressing which contains boric acid and tragacanth. This dressing is especially useful for treating wounds which contain multiresistant *Pseudomonas spp.* The boric acid lowers the pH of the wound and has been proven to be very effective against *Pseudomonas spp.* (Nagoba et al., 2013). Tragacanth is a natural poultice agent which has

been seen to stimulate wound healing in rats (Fayazzadeh et al., 2014). This dressing can be applied dry or wet depending on the amount of exudate produced by the wound. Stashak (2008c) recommends the use of this dressing for infected hoof wounds.

In the inflammatory phase honey can be used to reduce the bacterial load and to mitigate the chronic inflammation, so healthier granulation tissue is formed. However, the evidence supporting the anti-bacterial effect of honey is variable and depends on the product used (Vandamme et al, 2013). If honey is used for wound treatment, it is recommended to use a gamma radiated medical grade honey (e.g. L-mesitran™, Klinion; Revamil™, BiologiQ; Medihoney™, Derma Sciences, …), since commercial honey can contain (an)aerobic bacteria (Al-Waili et al, 2012; Carnwath et al., 2014). The use of honey in veterinary medicine will be discussed further in chapter 1.3 'An overview of the renewed interest in older therapies and the latest developments in equine wound management'.

Hydrogels contain 90 to 95 % water and are available as a sheet (Flexigel™, Smith & Nephew; NuMed Hydrogel dressing sheet™, Numed Industries, …), amorphous gel (Intrasite Gel™, Smith & Nephew; Flamigel™, Flen Pharma…) or an impregnated gauze (Kendall hydrogel impregnated gauze™, Covidien; DermaGauze™, DermaRite…) (Stashak, 2008c). In horses the sheets have been shown to stimulate exuberant granulation tissue, just as hydrocolloid dressings (Tegaderm Hyrdocolloid™, 3M…) (Stashak, 2008c). Therefore, in the authors institution only the amorphous gel form is used for wounds which cannot be covered with a bandage. The hydrogel prevents the wound from desiccating and creates a moist wound environment which stimulates wound healing.

Fibroproliferative phase

In the fibroproliferative phase, granulation tissue is formed to fill the defect. During this phase, it is the goal of a treatment to stimulate the formation of healthy granulation tissue (smooth and pink appearance without clefts or grooves) without creating exuberant granulation tissue.

To stimulate granulation tissue formation, calcium alginate dressings (e.g. Curasorb™, Kendall) are frequently used (Hendrickson, 2002). These dressings are especially useful in wounds were bone is visible or very deep wounds (Fig. 7). The dressing can be applied dry or wet depending on the amount of exudate produced by the wound (dry dressing to a wet wound or a wet dressing to dry wound). When the dressing absorbs the wound fluid, it turns into a gel promoting a moist wound environment (Stashak, 2008c) (Fig. 8). Moreover, the dressing does not adhere to the wound surface which makes it easy and painless to remove during a bandage change. The calcium alginate dressing is best applied if wound debridement is no longer necessary, since, to the author's experience, this type of dressing

may not sufficiently inhibit a developing infection. Therefore, if there is fear for progression to an infection, but the defect has to filled immediately (e.g. exposed hardware, proximity of body/synovial cavity...), a silver impregnated calcium alginate dressing can be used (e.g. Suprasorb A+ Ag^{TM}, Lohmann & Rauscher).

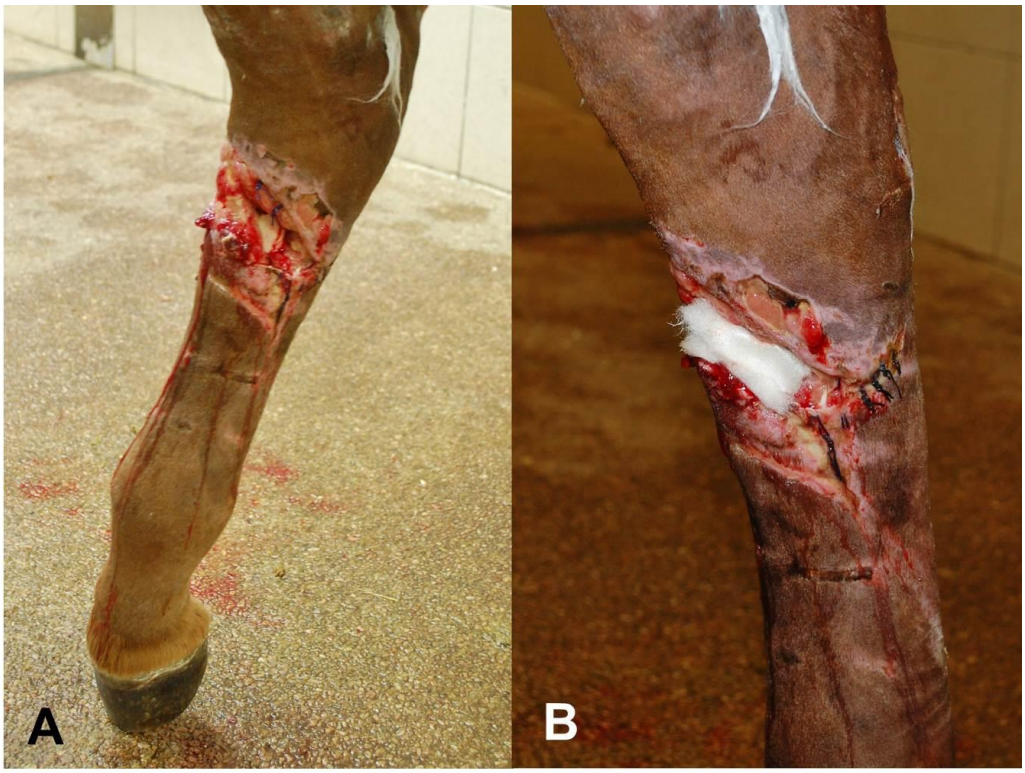


Figure 7. (A) A second intention healing wound with an irregular bed of granulation tissue and exposed bone. (B) To stimulate granulation tissue formation and cover the exposed bone, the wound defect is filled with a calcium alginate dressing.

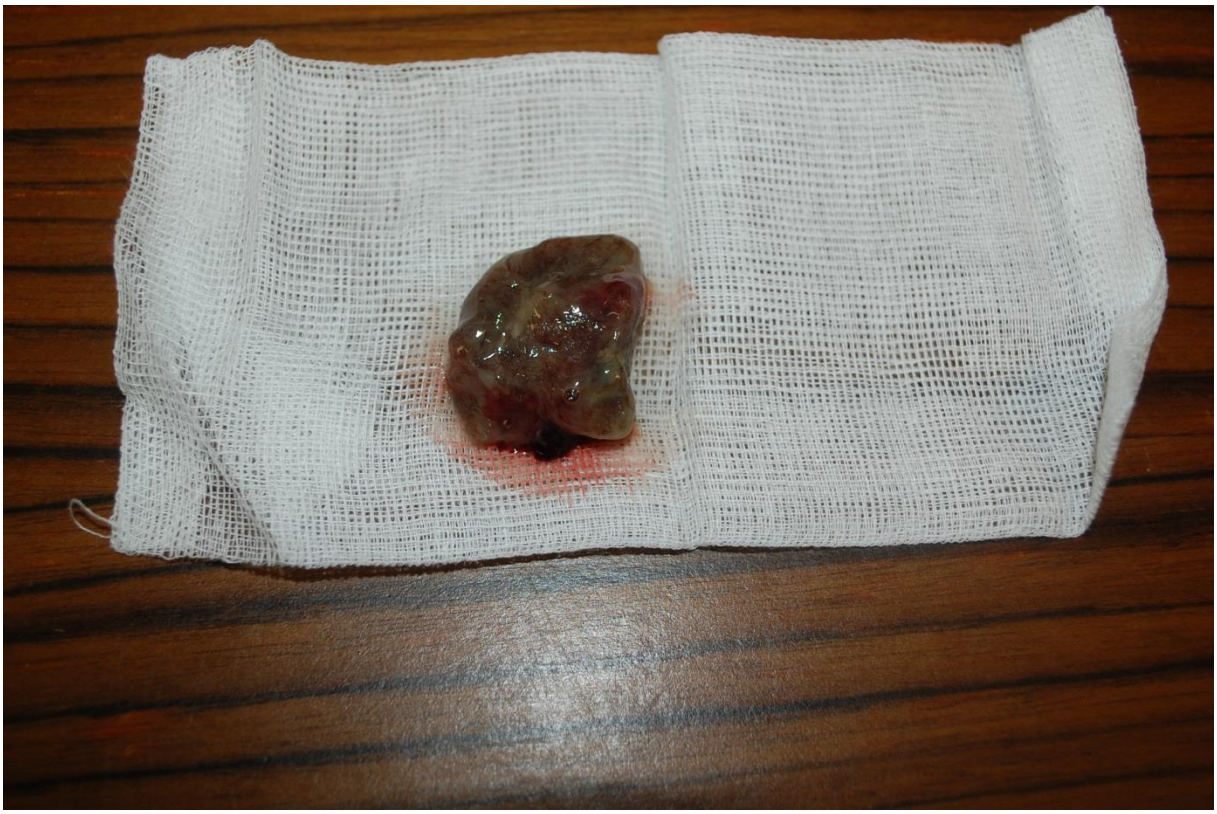


Figure 8. A calcium alginate dressing, which has been removed from the wound bed after three days.

The dressing has absorbed the wound fluid and has turned into a gel, promoting a moist wound environment and making it easy to remove at a bandage change.

Another method to stimulate granulation tissue formation is the use of negative pressure wound therapy (NPWT). This therapy uses a specialized type of airtight bandage and is proven in men to stimulate granulation tissue formation (Mouës et al., 2011). In veterinary medicine, research on this type of wound treatment is limited. The mechanisms of action of negative pressure wound therapy, its indications and the practicalities will be discussed further in chapter 1.4 ‘Negative pressure wound therapy: what does it entail?’.

Contraction and epithelialization phase

When a healthy bed of granulation tissue is formed, the new epithelium has to grow over the wound bed to close the wound. This process can be stimulated with the use of topical treatments.

Semi-occlusive hydrophilic polyurethane foams (e.g. hydrophilic foam dressing™, Kendall; Allevyn™, Smith & Nephew; Mepilex™, Mölnlycke health care…) are absorbent and non-adherent. They are vapour permeable, easy to contour to the wound surface and are removed easily without disturbing the healing tissue (Stashak, 2008c). Excess wound fluid is

absorbed into the dressing, but a moist wound environment is still preserved, thus promoting wound healing and epithelialization (Stashak, 2008c). This type of dressing is routinely used in our institution for wounds in the epithelialization phase after a healthy bed of granulation tissue is formed (Fig. 9). The dressing is left in place for three to five days, sometimes seven days if the wound is little exudative. These types of dressings are also available impregnated with silver (e.g. Allevyn Ag™, Smith & Nephew; Mepilex Ag™, Mölnlycke health care…) to reduce bacterial bioburden. However, since silver ions also have a certain cytotoxic effect, their use should be limited to wounds with signs of infection (Toy and Macera, 2011). A study on the efficacy of a silver impregnated foam dressing will be discussed further in chapter 1.3 'An overview of the renewed interest in older therapies and the latest developments in equine wound management'.

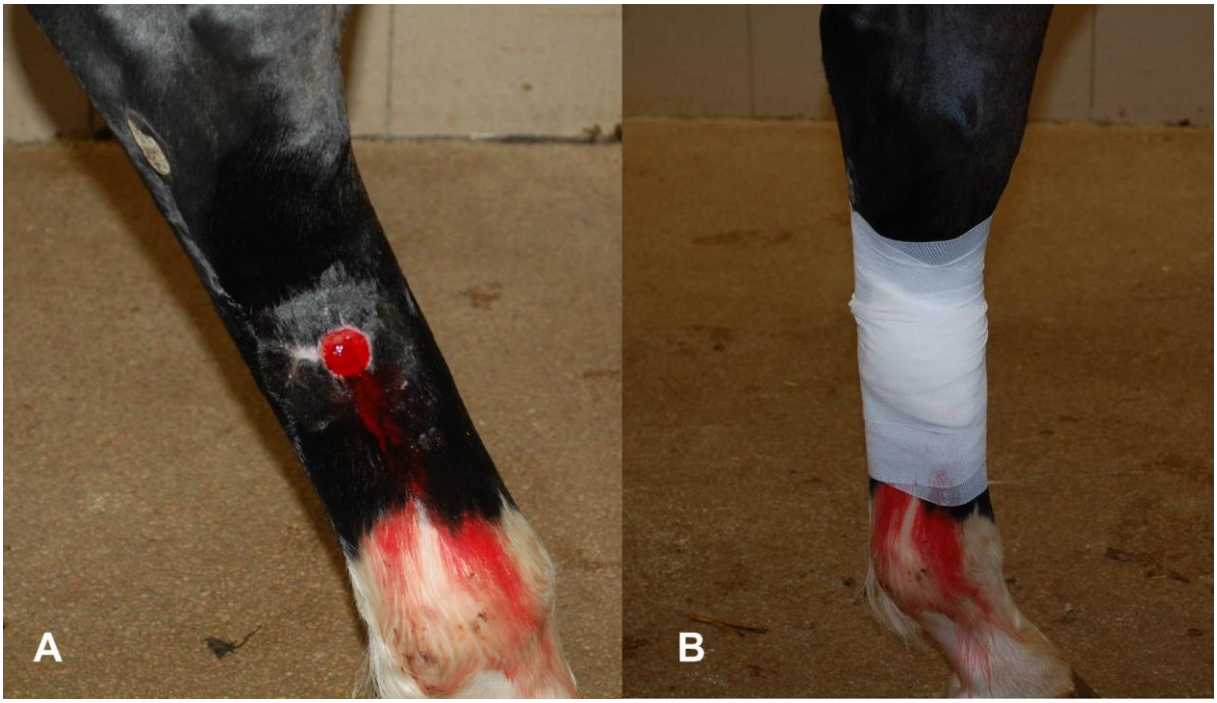


Figure 9. (A) A second intention healing wound with a rather dark, but overall healthy bed of granulation tissue. (B) To stimulate the epithelialization and promote a moist wound environment the wound is dressed with a semi-occlusive hydrophilic polyurethane foam, which is kept in place with an elastic retention dressing

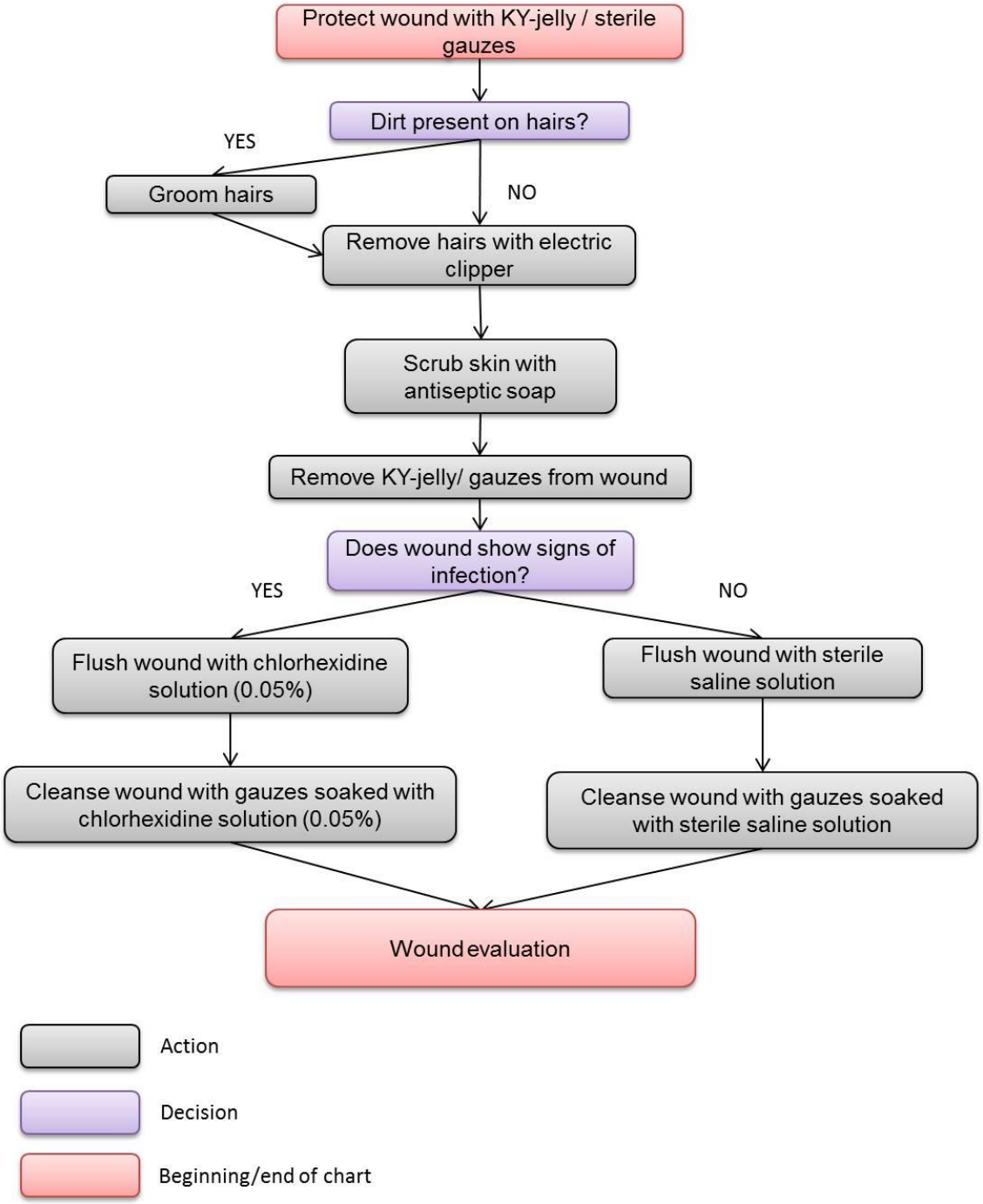
Honey can also be used in this stage of wound healing, since honey lowers the pH of the wound surface and aids epithelialization (Gethin et al., 2008). Moreover, if low quality granulation tissue fills the wound at this stage, honey can also lower the bacterial burden and potentially improve wound healing. In the author's institution, gamma radiated medical grade honey ointments (e.g. L-mesitran™, Klinion) combined with a semi-occlusive hydrophilic

polyurethane foam are frequently used in this stage of healing if the wound bed appears of low quality and potentially infected.

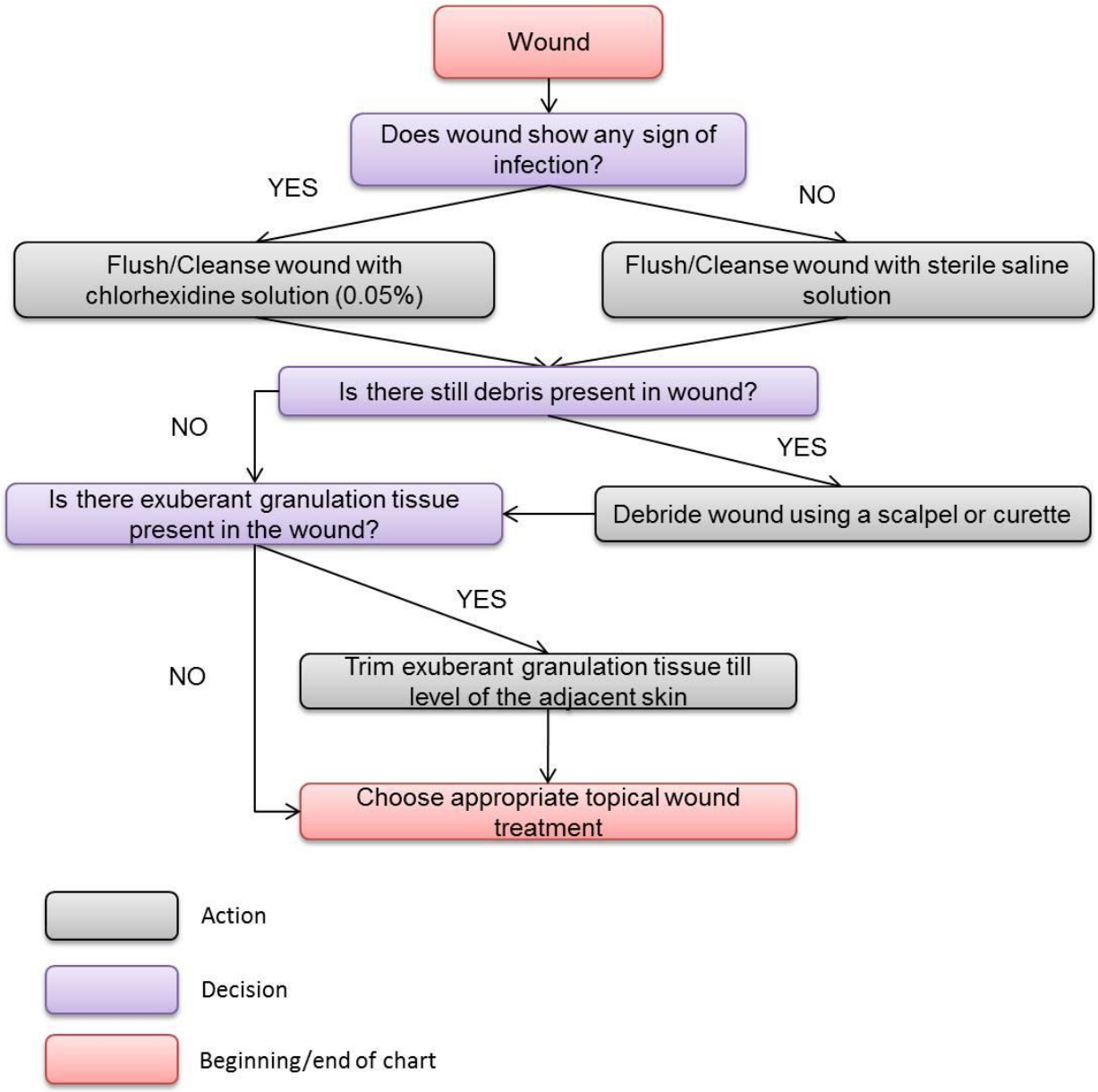
5 STANDARD OF CARE FLOW CHART

Below, a summary is given of the guidelines described above, by means of flow charts.

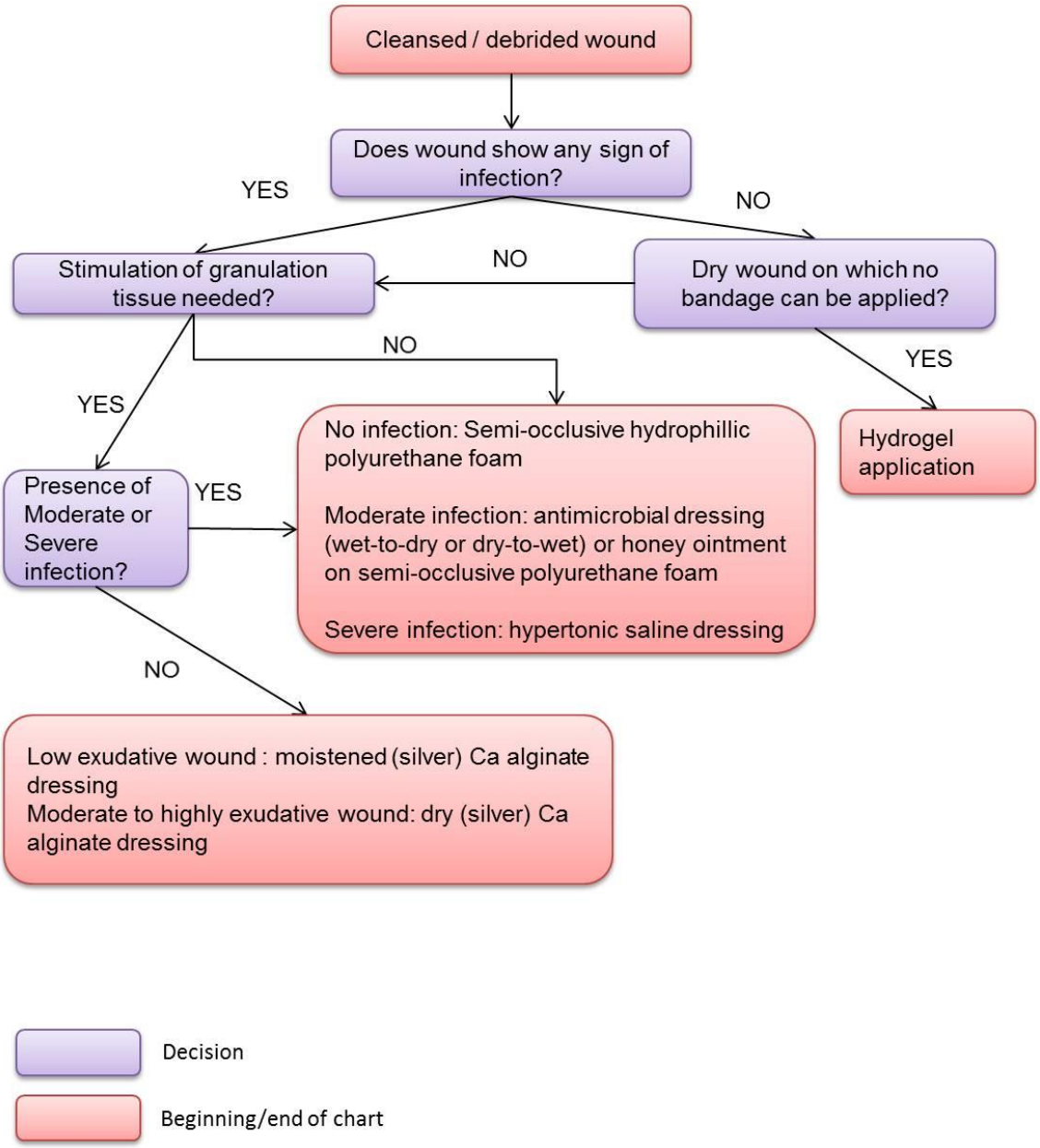
Flow chart 1. A step-by-step guide to the wound preparation process.



Flow chart 2. A step-by-step guide to wound debridement.



Flow chart 3. A step-by-step guide to the right choice of a topical wound treatment



AN OVERVIEW OF THE RENEWED INTEREST IN OLDER THERAPIES AND THE LATEST DEVELOPMENTS IN EQUINE WOUND MANAGEMENT

1 INTRODUCTION

Because of the fright-and-flight instinct of horses, traumatic wounds are very common. These wounds are cause of noteworthy morbidity and even mortality in horses (Theoret et al., 2016). If the wounds are located at the distal limb, the wound healing is often complicated and delayed, which increases the cost for the owner (Theoret et al., 2016). Therefore, there is a continuing search for treatments which can expedite wound healing, especially at the equine distal limb. In this part of chapter 1 an overview will be given, not only of the newest developments in equine wound management, but also of some older treatments which have gained renewed interest for the management of second intention healing distal limb wounds.

2 OLD THERAPIES WHICH RECEIVE RENEWED INTEREST

Honey

Honey is in fact an old method for wound treatment, but is gaining renewed interest over the years because of the growing problem of antimicrobial resistance (Vandamme et al., 2013). The antibacterial capacity of honey lies in a combination of several mechanisms. When honey comes in contact with wound exudate hydrogen peroxide is formed by the enzyme glucose oxidase, which is inherently present in honey. Moreover, honey is an acid product (pH 3.2-4.5) and has a high osmolarity, which further inhibits bacterial growth. Certain types of honey such as Manuka honey (e.g. Medihoney^{TM}, Derma Sciences) possess an additional antibacterial effect apart from these mechanisms and are therefore very coveted for wound treatment applications (Vandamme et al., 2013). *In* vitro, honey has been reported to inhibit the growth of about 60 species of bacteria including aerobes and anaerobes, Gram positives, and Gram negatives (Mohapatra et al., 2011). However *in vivo*, the evidence for the antibacterial effect of honey is not consistent and depends on the type of wound and the product used (Vandamme et al., 2013). In contrast, the wound healing stimulating properties of honey are considered to be sufficiently evidence based in human medicine (Vandamme et al., 2013).

In equine medicine, an *in vitro* study has been performed on the antibacterial effect of different types of honey products on several bacteria isolated from equine wounds (Carnwath et al., 2014). Eighteen of the 29 tested honey products were contaminated with aerobic bacteria or fungi. Of the remaining 11 honey products, eight were effective against all ten bacterial species at concentrations varying from < 2% to 16% (v/v). Carnwath et al. (2014) stated that Scottish Heather honey had the best antibacterial effect and was efficient at lower concentrations than the Manuka honey products. Additionally, several *in vivo* studies have been performed on the use of Manuka honey on second intention wound healing in horses (Bischofberger et al. 2011, Bischofberger et al., 2013, Bischofberger et al., 2016). Bischofberger et al. (2011) performed a preliminary study on the effect of Manuka honey on contaminated second intention healing distal limb wounds in eight horses. They reported that Manuka honey decreased wound retraction and that the Manuka honey treated wounds were significantly smaller than the untreated control wounds until day 42. However, the overall healing time was not significantly different between the two treatments groups. In continuation of this study, Bischofberger et al. (2013) investigated the long and short term effect of Manuka honey on second intention healing distal limb wounds in horses. They tested two different formulations of Manuka honey and two different treatment periods. The application of Manuka honey gel throughout the healing period gave the fastest wound

healing compared to all the other treatment protocols. Manuka honey and Manuka honey gel application for 12 days gave a significantly faster wound healing compared to gel control and untreated control wounds. In a follow up study, Bischofberger et al. (2016) investigated more clearly the antibacterial effect of Manuka honey gel and its microscopical effect on wound healing in ten horses. Interestingly, Manuka honey gel had no significant effect on bacterial counts or on transforming growth factors β1 and β3 (TGF-β1 and TGF-β3). However, Manuka honey gel did reduce wound inflammation, increased neovascularisation, fibrosis and collagen orientation and stimulated epithelial hyperplasia.

Based on these studies, Manuka honey seems promising but all the studies were performed by one research group and the wound model used were rather small wounds. Further research is needed to gain more pronounced evidence of the effects of honey on equine second intention wound healing.

Maggot debridement therapy

The most common maggots used for debridement in veterinary medicine are the larvae of the green bottle fly *Lucilia sericata.* Their saliva is rich of digestive enzymes such as collagenases, trypsin-like and chymotrypsin-like enzymes, which enables them to breakdown necrotic tissue and ingest it as a liquid (Jones and Wall, 2008). The use of maggots as a debridement technique is an ancient method, but has regained renewed interest with the growing problem of antimicrobial resistance in micro-organisms (Jones and Wall, 2008; Lepage et al., 2014). The secretory products of maggots have an antibacterial effect, which is useful against multi-resistant bacteria (Jones and Wall, 2008).

Currently, to the author's knowledge, the literature on maggot debridement therapy in horses only consists of case reports or surveys. Morrison (2007) reported on the use of maggot debridement therapy for foot infections in the horse. The case series consisted of horses treated for coffin bone osteomyelitis, chronic laminitis, septic navicular bursa, chronic distal interphalangeal joint sepsis, hoof canker, acute caudal coffin bone rotation, non-healing foot ulcers and necrosis of the collateral cartilage. Maggot debridement therapy was often used after a light surgical debridement and in addition to general antibiotics or regional limb perfusion. Morrison (2007) found maggot debridement therapy a useful minimal invasive method to debride hoof injuries. Sherman et al. (2007) conducted a survey on the use of maggot debridement therapy by US practitioners. They reported that no adverse events were encountered during the therapy by the practitioners and that maggot debridement therapy was a useful method to debride wounds on anatomically difficult locations or for wounds unresponsive to conventional therapy. In this survey, a case series of 13 horses was included. In 12 of the 13 horses the infection in the wounds was controlled or eradicated.

More recently, Lepage et al. (2012) described a case series of 41 equids in which maggot debridement therapy was used. They reported signs of discomfort in seven horses, but no serious adverse events. Only in five cases a second application of maggots to the wound was necessary. Eradication of infection, debridement and healing of the wound occurred in all but three cases. In two of these cases a malignant process was present in the wound, while in the third case a sequester was seen on radiography. Lepage et al. (2012) recommended from their personal experience to use free range maggots instead of maggots in a biobag for wound debridement. They concluded that that maggot debridement therapy should be considered after conventional therapies fail, considering the cost of the therapy.

There are no randomized controlled experimental or clinical studies available on maggot debridement therapy in horses. Therefore, further research is needed to objectively assess the effect of maggot debridement therapy for equine second intention wound healing.

Negative pressure wound therapy

Negative pressure wound therapy (NPWT) is an established method for wound treatment in human medicine and has been shown to enhance healing in a wide variety of wounds (Birke-Sorensen et al., 2011; Krug et al., 2011; Vig et al., 2011; Miller, 2014; Huang et al., 2014; Howe, 2015; Dumville et al., 2015). NPWT uses a specialized type of bandage to exert negative pressure to the wound surface (Huang et al., 2014). This negative pressure enhances wound healing by stimulating granulation tissue formation and increasing local blood flow (Morykwas et al., 1997). Currently, NPWT is gaining increased interest in the field of veterinary medicine because of its promising results in human medicine (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Demaria et al., 2011; Jordana et al., 2011; Cioffi et al., 2012; Stanley et al., 2013; Pitt and Stanley; 2014; Nolff et al., 2015; Howe et al., 2015). In horses, NPWT is used to either stimulate granulation tissue formation during the fibroproliferative phase of the wound, or to enhance graft acceptance after skin grafting (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Jordana et al., 2011). Nevertheless, the overall body of research on NPWT is scarce in horses, and studies are needed to elucidate the potential of the NPWT for equine medicine. This old type of wound treatment that received a renewed interest over the last decades will be the main subject of this PhD study. A literary review on the mechanisms of action of NPWT, its indications, the history and its use in horses will be given below.

3 LATEST DEVELOPMENTS IN EQUINE WOUND MANAGEMENT

Silver impregnated semi-occlusive foam

The semi-occlusive hydrophilic polyurethane foam is a dressing which provides a moist wound environment and stimulates epithelialization and wound healing (Stashak, 2008c). However, since wound infection is an important cause of delayed wound healing, additional measures sometimes are necessary to reduce bacterial load (Stashak, 2008b). Silver ions are known to decrease bacterial load *in vitro* and have become increasingly popular in wound dressings to manage infected or contaminated wounds (Lansdown, 2004; Toy and Macera, 2011). However, the antibacterial effect of some of these dressings and their effect on wound healing remains to be elucidated.

Kelleher et al. (2015) investigated the application of a silver sodium zirconium phosphate polyurethane foam dressing on second intention healing distal limb wounds in horses. They found that the silver foam significantly decreased the wound area when compared to the control/reference therapy (a non-adherent, absorbent abdominal pad) over the first 30 days of the study period and over the entire study period of 60 days. However, the overall healing time was not significantly different between the two treatments. Additionally, the wound area was not significantly different between the two treatments during the second half of the study period (30-60 days). The silver foam also significantly decreased granulation tissue scores (less granulation tissue formation) when compared to the control/reference therapy during the first half of the study period and over the entire study period. A big limitation of this study is the lack of a quantitative follow up of the bacterial growth with the two treatments. Only qualitative bacteriology was performed, making the study inadequate to assess the antibacterial effect of the silver foam. This is regrettable, since the main indication to use a silver impregnated wound dressing is to reduce the bacterial load and hence lowering or eradicating wound infection. To the authors' knowledge, this study is the only one to test a silver impregnated wound dressing in horses.

Topical oxygen therapy

Oxygen is an indispensable molecule for a normal wound healing process. Oxygen is necessary for a normal collagen disposition, developing sufficient tensile strength, a strong respiratory burst of the phagocytic cells, induction of epithelialization and it has an influence on several growth factors (Gordillo and Sen, 2009). However, in a wound, blood supply is decreased due to thrombosis, swelling, and tissue loss. This diminished blood supply results in hypoxia, which slows down wound healing (Tracey et al., 2014). To counteract hypoxia, extra oxygen can be applied onto the wound surface by using topical oxygen therapy. With

this therapy, oxygen is delivered to the wound bed by means of a small cannula, which is placed beneath the primary dressing and connected to a small electrochemical oxygen concentrating device attached to the body. Topical oxygen therapy is of interest for chronic non healing wounds with hypoxia such as pressure ulcers or diabetic wounds in humans (Gordillo and Sen, 2009). Additionally, this topical oxygen therapy can also be interesting for wound treatment in horses, because of the low oxygenation and high occlusion of microvessels in equine distal limb wounds (Lepault et al., 2005; Celeste et al., 2011).

Tracey et al. (2014) performed a study on the effect of topical oxygen therapy on distal limb wounds in horses. They reported that topical oxygen therapy did not significantly decrease mean healing time compared to the control/reference therapy. They also mentioned that there was no influence of topical oxygen therapy on histological inflammation, angiogenesis, epithelial hyperplasia and fibroplasia compared to the control/reference therapy. The bacterial growth in this study was not assessed quantitatively, only qualitatively. Thus, the effect of topical oxygen therapy on the bacterial load in the wounds could not be assessed. The study was performed on the front and hind limbs of four horses. Tracey et al. (2014) did find a difference in wound healing between front and hind limbs of horses, so this has to be considered when developing a study design on distal limb wound healing in horses.

To the author's knowledge, the study of Tracey et al. (2014) is the only that has been performed on the use of topical oxygen therapy for wound healing in horses.

Biological therapies

Platelet rich plasma

Platelets are a rich source of growth factors which are involved in the natural healing process of a wound and are thus an interesting option to enhance wound healing (Carter et al. 2003, DeRossi et al., 2009). External platelets are usually applied to the wound surface under the form of platelet rich plasma (PRP). The platelets are concentrated in the plasma by specific centrifugation of a blood sample, so when the PRP is applied to a wound a high amount of growth factors are delivered to the wound surface (Carter et al., 2003, Monteiro et al., 2009). PRP has to be activated by adding thrombin, which stimulates the platelets to release their granules filled with growth factors and the PRP to form a gel so it can be more easily applied to a wound (Carter et al, 2003, Monteiro et al., 2009).

In equine medicine, the reported effects of PRP on wound healing are variable and the grade of evidence is low. Carter et al. (2003) and DeRossi et al. (2009) both found that PRP increased epithelial differentiation and stimulated the formation of more organized collagen bundles in scar tissue compared to the control wounds, in second intention (Carter et al.,

2003) and first intention healing wounds (DeRossi et al., 2009). However, the results of Carter et al. (2003) were based on distal limb wounds in only one horse. Monteiro et al. (2009) performed a more substantiated study and created open wounds on the metacarpi of 6 horses. They found that PRP did not accelerate wound healing. On the contrary, PRP delayed wound healing at week one, two and three after surgery. PRP also stimulated the formation of exuberant granulation tissue. More recently, Maciel et al (2012) used PRP on an equine burn wound model on flanks of horses. They found that PRP accelerated repair and induced fibrosis. However, only two horses were included per treatment group.

Due to the great variability in study design and the low number of animals used in studies on PRP, it is hard to formulate a recommendation. More research on PRP is necessary to fully assess the effect of PRP on equine second intention wound healing.

Stem cells

Stem cells are currently a very popular domain for research in both human and veterinary medicine (Volk and Theoret, 2013; Borena et al., 2015). Stem cells are unspecialized cells which can differentiate into other cell types and self-regenerate. They can be categorized based on their ability to differentiate into different cell types (totipotent, pluripotent, multipotent or progenitor cells) or based on their origin (embryonic stem cells, umbilical cord stem cells, adult stem cells) (Borena et al., 2015). Thus, there are several types of stem cells, each with their own potential to treat certain pathological conditions (Volk and Theoret, 2013; Borena et al., 2015). In this PhD study, only the use of stem cells for equine wound healing will be discussed.

For equine wound healing mainly 2 cell types are used: mesenchymal stem cells (MSCs) and epithelial-like stem/progenitor cells (EpSCs). MSCs are adult multipotent stem cells which can be found in the mesodermal tissues and can differentiate into osteoblasts, chondroblasts, adipocytes, tenocytes and myocytes (Borena et al., 2015). In veterinary medicine, they are often harvested from the bone marrow, fat tissue, peripheral blood and umbilical cord blood (Volk and Theoret, 2013). EpSCs are adult progenitor cells and are able to differentiate in epidermal cells. They can be harvested from the epidermis and the hair follicles (Borena et al., 2015).

Research on the use of stem cells for equine wound healing is limited. Iacone et al. (2012) described in a case study that the combination of amniotic fluid MSCs and PRP induced significantly faster wound healing of a decubitus ulcer in a septic neonatal foal compared to PRP alone or aloe gel. In a preliminary study, Broeckx et al. (2014) investigated the effect of a combination EpSCs and PRP on second intention wound healing on body wounds in a

horse. They found that the combination of stem cells and PRP significantly decreased wound surface area compared to a treatment with PRP only. They also reported that the EpSCs+PRP treated wounds showed less pronounced granulation tissue formation and a more mature replacement tissue which more closely resembled the original skin. However, only one horse was used in this study. Spaas et al. (2013) described a case series of four horses whose open wounds were locally treated with peripheral blood stem cells. The wounds were all located at the distal limb and were not responsive to conventional therapy for three months. After treatment with the peripheral blood stem cells, almost all wounds developed granulation tissue within two weeks. However, the period for total wound healing after the stem cell application was still a mean of 160 days and there was no control/reference group. More recently, Broeckx et al. (2015), performed a randomized controlled experimental trial on five horses to investigate the effect of autologous and allogenic EpSCs on second intention healing body wounds compared to untreated and vehicle treated (only medium) control wounds. They found that over the entire follow-up period the autologous EpSCs treated wounds had a significantly smaller mean wound circumference and mean surface area compared to vehicle treated wounds. The EpSCs treated wounds also displayed a higher neovascularization and thinner granulation tissue formation.

Thus, only one study on the use of stem cells in equine second intention wound healing was a controlled trial. The rest consisted of case reports, or were performed on only one experimental horse. Further research on stem cells is therefore needed to formulate a recommendation on their use for equine wound healing.

NEGATIVE PRESSURE WOUND THERAPY: WHAT DOES IT ENTAIL?

1 THE HISTORY OF NEGATIVE PRESSURE WOUND THERAPY AND ITS USE IN HUMAN MEDICINE

Introduction

Negative pressure wound therapy is a wound treatment technique which applies negative pressure to a wound surface to enhance healing (Dumville et al., 2015). NPWT as we know it today has become popular since the publications of Morykwas et al. in late 1990's (Morykwas et al., 1997; Argenta and Morykwas, 1997; Morykwas et al., 1999). However, treating wounds with negative pressure is an ancient technique. Descriptions of its use date back to as early as the Roman era (Danino and Weber, 2007; Miller, 2014). In those days, modern tubing, pump systems and fluid collection cups were not available, so people used their mouths to create negative pressure at the wound site. The person treating the patient applied his mouth directly over the wound and sucked out all the blood cloths, pus, and necrotic tissue (Miller, 2014). This proved to be a successful technique in ancient times and was thus frequently applied. In replacement of having to use the mouth, 'cupping glasses' were developed for sucking fluid out of a wound. These little glass domes were first heated and then placed over the wound in contact with the patient's skin (Miller, 2014). When the dome cooled down, the suction force on the wound increased (Miller, 2014). These cupping glasses were the main technique for applying negative pressure therapy throughout the 19th century (Miller, 2014). In the 20th century, the Russians developed a more advanced system of NPWT. They placed a flexible transparent cover on the wound which was connected through tubing to a wall suction system. This method of negative pressure therapy also included fluid collection cups (Danino and Weber, 2007). Chariker et al. (1989) were the first to describe a NPWT system using a wound filler. In this publication, a gauze dressing was applied in the wound, covered with an adhesive transparent film and connected with a silicon drain to a wall suction system generating approximately 60-80 mm Hg negative pressure. This technique became known as the Chariker-Jeter technique. Not long after this publication Morykwas et al. (1997), published their work using a reticulated open cell polyurethane foam as a wound filler. This NPWT system was patented by the company KCI medical Inc. under the name of Vacuum Assisted Closure (V.A.C.) and was the first of the modern NPWT systems as we known them today.

Currently, different modern NPWT systems are available. These include, but are not limited to:

- RENASYS™ (Smith & Nephew)
- V.A.C.™ (KCI medical Inc.)

- extriCare™ (Devon medical products)
- INVIA™ (Medela)
- Suprasorb CNP™ (Lohman-Rauscher)
- Avance™ (Mölnlycke health care)
- Venturi™ (Talley group Ltd.)
- …

The V.A.C. product line of KCI medical Inc. and the NPWT systems of Smith and Nephew are currently the most commonly used systems (FDA, 2011). Additionally, most of the research on NPWT has been performed with the V.A.C. product line (Mouës et al., 2011).

Every NPWT bandage consists of a couple of basic elements which can vary slightly in appearance and composition between the different brands (Fig. 10). The first basic element is a wound filler, which can be a foam dressing or a gauze dressing depending on the used brand. The second basic element is an occlusive drape, which is applied over the wound dressing to create an airtight seal of the wound. The third basic element is an evacuation tube, which connects the wound dressing to the negative pressure system. This tube transfers the negative pressure from a pump (the fourth basic element) to the wound dressing and evacuates excess wound fluid. The fourth basic element, namely the negative pressure pump, generates the negative pressure and also contains a fluid collection canister.

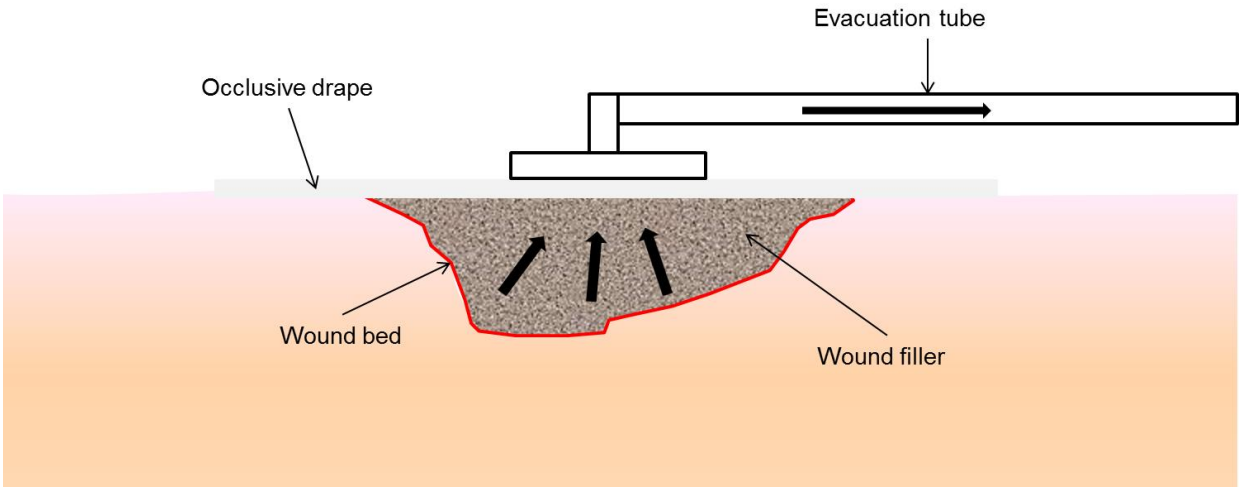


Figure 10. A schematic overview of the basic elements of a negative pressure wound therapy bandage.

The wound bed is filled with a wound filler, usually consisting of a foam dressing. This dressing is held in place and sealed airtight with a transparent occlusive drape. An evacuation tube is applied to connect the wound filler with the negative pressure pump (not visible on this figure). Through this tube excess wound fluid is evacuated and the negative pressure is delivered to the wound filler.

There are different types of wound fillers which can be used with NPWT. The Chariker-Jeter technique, patented by Smith & Nephew, uses gauze dressings to fill the wound cavity and evenly distribute the negative pressure to the wound bed. These gauze dressings are impregnated with polyhexamethylene biguanide (PHMB) and thus have an additional antibacterial activity. However, most of the research on NPWT has been performed with foam dressings as a wound filler (Mouës et al., 2011).

Negative pressure wound therapy: mechanisms of action, indications and contraindications in human medicine

In literature, several mechanisms of action have been attributed to NPWT (table 2). However, not all these mechanisms of action have a solid evidence based foundation. Mouës et al. (2011) performed a review to investigate the amount of evidence available for each of these mechanisms of action. They found that there were no studies which objectively assess the amount of moisture in the wound for NPWT nor any other occlusive dressing. They also mentioned that there are two studies who report a reduction of oedema with NPWT. However, the inclusion numbers in these studies were low. Based on the current literature, Mouës et al. (2011) stated that there is sufficient evidence to confirm the increase in local blood flow, stimulation of angiogenesis, stimulation of granulation tissue formation and cell proliferation with NPWT. They also concluded that NPWT reduced wound size and complexity in certain types of wounds (chronic wounds) and that there are indications that NPWT modulates the matrix metalloproteinases in the wound fluid. Concerning bacterial reduction, Mouës et al. (2011) reported that this subject was still controversial and more research was needed.

Table 2. An overview of the claimed mechanisms of action of NPWT in current literature (Mouës et al. 2011).

- Creates a moist wound environment
- Reduces oedema
- Increases local blood flow
- Stimulates angiogenesis
- Stimulates formation of granulation tissue
- Stimulates cell proliferation
- Reduces size and complexity of a wound
- Removes excess exudate and soluble healing factors from wound
- Reduces bacterial load

Despite the lack of evidence on the mechanisms of action of NPWT, in human medicine it is a widely implemented wound treatment technique used to enhance healing in a variety of open wounds (table 3). Recently, NPWT has also been used prophylactically for the management of closed surgical incisions to reduce surgical site infections (Semsarzadeh et al., 2015). However, NPWT may never be used as a replacement for surgical debridement of the wound (Krug et al., 2011).

When considering NPWT as treatment some contraindications have to be borne in mind (table 3). NPWT should not be applied on a bleeding wound or used for a patient with a clotting disorder, since the suction force of the system can cause a rapid amount of blood loss. In some commercial systems, the fluid canisters can only hold a limited amount of fluid (e.g. 300-500 mL) and an additional alarm sounds if the fluid canister is full, so excessive blood loss can be prevented (Willy, 2006). NPWT should not be applied on exposed blood vessels or vascular anastomoses, since erosion of these structures can occur with bleeding and a comprised vascularisation as a consequence (Howe, 2015). When NPWT is applied to a wound containing a malignant process, it can stimulate the tumour progression and if it is applied after excision of a malignant process, it can stimulate recurrence of the tumour (Willy, 2006; Howe, 2015). NPWT should also not be applied if necrotic tissue or an untreated osteomyelitis is present in the wound. The necrotic tissue prevents new healthy tissue formation and NPWT is not efficient in treating large infection sites (Willy, 2006).

Table 3. The most common indications and contraindications for NPWT in humans.

Indications:

- Soft tissue traumatic wounds and open fractures (Krug et al., 2011)
- Partial thickness burn wounds (Krug et al., 2011)
- Skin grafts (Krug et al., 2011, Mouës et al, 2011)
- Chronic wounds including (Vig et al., 2011):
    - Pressure ulcers
    - Diabetic foot ulcers
    - Venous leg ulcers
    - Ischaemic lower limb wounds
- Abdominal wall wounds (Mouës et al., 2011)

Contraindications (Willy,2006: Howe; 2015):

- The presence of clotting disorders in the patient
- Bleeding of the wound
- Exposed vessels and vascular anastomoses
- A malignant process in the wound
- Necrotic tissue present in the wound bed
- Untreated osteomyelitis

In human medicine, recommendations have been proposed for the use of NPWT (Birke-Sorensen et al., 2011). Concerning the choice of wound filler (gauze dressing or PU), this consensus study states that PU and gauze dressing both equally distribute the negative pressure to the wound bed. Additionally, no difference in stimulation of blood flow or wound contraction could be found for small wounds. However, for large wounds PU gives more wound contraction than gauze. Overall healing time and rate of reduction of wound size is not significantly different between PU and gauze dressings. Birke-Sorensen et al. (2011) also recommended to use a negative pressure within the range of -40 mm Hg to -150 mm Hg. Within this range micro-deformation (deformation of the individual cells) at the level of the wound surface occurs and there is an increase in blood flow. Additionally, this pressure range also ensures a maximal wound contraction, granulation tissue formation and excess wound fluid removal.

Pain has been reported as an issue when applying NPWT in human medicine (Birke-Sorensen et al., 2011). However, randomized controlled trials investigating the amount pain

experienced during NPWT compared to a standard dressing are scarce. Upton and Andrews (2015) mentioned the pain perceived during NPWT could be depending on the type of wound filler used, the stage of NPWT therapy and the type of NPWT system used. However, the evidence supporting these hypotheses is weak and mainly consist of non-controlled clinical trials. Nonetheless this lack of evidence, NPWT using a gauze dressing or a polyvinyl alcohol foam as a wound filler is recommended as an alternative to NPWT using a polyurethane foam to reduce pain during NPWT (Birke-Sorensen et al., 2011). Additionally, for this same purpose lower pressure levels are sometimes also recommended (Birke-Sorensen et al., 2011).

2 NEGATIVE PRESSURE WOUND THERAPY IN HORSES

The wound healing in the distal limb of horses is aberrant and shows similarities with chronic non-healing wounds in humans such as pressure ulcers and diabetic ulcers (Diegelmann and Evans, 2004; Theoret and Wilmink, 2013; Baltzis et al., 2014; Wilmink, 2014). The inflammatory response in these distal limb wounds has a slow onset, does not peak efficiently and persists over time leading to chronic inflammation and impeded wound healing (Wilmink, 2008). Moreover, due to this chronic inflammation the wound debridement is improficient, which increases the chance of wound infection (Wilmink, 2008). Wound healing is even further impaired because of a decreased oxygenation and higher occlusion of microvessels in distal limb wounds, which contribute to the chronic inflammation and exuberant granulation tissue formation (Lepault et al., 2005; Celeste et al., 2011). NPWT, which is frequently used in human medicine to treat chronic wounds (Vig et al., 2011), could potentially address certain of these issues. NPWT would aid wound healing in equine distal limb wounds, since it increases local blood flow, stimulates angiogenesis, granulation tissue formation and cell proliferation. It also reduces size and complexity of a wound and there are indications that NPWT modulates the inflammatory response (Mouës et al., 2011). However, until recently, research on NPWT in horses only consisted of case reports (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Jordana et al., 2011). Gemeinhardt and Molnar (2005) described the use of NPWT for the treatment of a traumatic neck wound with massive tissue loss in a horse. They reported that, to their opinion, the wound healed faster and with less scaring than was to be expected for that type of wound. Gemeinhardt and Molnar also compared the decrease in wound area of their case report to the results on evolution of wound area of second intention healing equine body wounds of Wilmink et al. (1999a). However, this comparison was based on an estimation of the results of Wilmink et al. (1999a). Moreover, they used the ruler technique (height x width) to measure the wound dimensions in their case report. This technique is not very accurate as it assumes a rectangular shape of the wound, which was not the case for the neck wound. Therefore, the grade of the evidence in this case is low. Rijkenhuizen et al. (2005) reported about the use of NPWT to enhance graft acceptance after Meek micrografting in 2 equine patients. A graft acceptance of at least 75% was seen in both cases. Additionally, they mentioned that NPWT provided an adequate wound drainage, a good fixation of the grafts to the wound bed and limited shear forces. However, as there was no control in this case report, the grade of evidence is again low. Jordana et al. (2011) also described the use of NPWT to enhance graft acceptance, but in contrast to Rijkenhuizen et al. (2005), NPWT was applied on a distal limb wound and punch grafts were used to close the wound. Jordana et al. (2011) reported a graft acceptance of nearly 100% and concluded that NPWT enhanced the healing of the

large chronic distal limb wound. Based on these case reports, NPWT seems promising for treating (large) wounds in horses. However, before the start of this PhD study, to the author's knowledge, controlled studies on NPWT in horses were lacking.

Further research on the effect of NPWT on the abnormal wound healing in the distal limbs of horses could also be potentially valuable for human medicine, as the horse can function as a large animal wound model for chronic non healing wounds in humans (Theoret and Wilmink, 2013, Wilmink, 2014). Indeed, well-designed randomized controlled trials on NPWT in human medicine are scarce and difficult to perform due to practical and ethical reasons (Vlayen et al., 2007). Moreover, despite there are several publications available in human medicine with recommendations for the use of NPWT in general and for specific types of wounds (Krug et al., 2011; Vig et al., 2011, Birke-Sorensen et al., 2011), a report of the Belgian federal healthcare knowledge centre stated that there is insufficient evidence to use NPWT as a routine treatment for chronic and acute wounds (Vlayen et al., 2007). Additionally, certain mechanisms of actions, such as reduction of bacterial load and modulation of inflammatory response, remain to be fully elucidated or further research is needed to confirm the current statements (Mouës et al., 2011; Patmo et al., 2014).

CLINICAL APPLICATION OF NEGATIVE PRESSURE WOUND THERAPY

1 INTRODUCTION

In equine medicine, the only research available on NPWT consists of case reports (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Jordana et al., 2011) and a guideline on how to apply NPWT in horses is lacking. In veterinary medicine in general, the application of NPWT bandages is mainly based on the knowledge extrapolated from human medicine (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Demaria et al., 2011; Jordana et al., 2011; Cioffi et al., 2012; Stanley et al., 2013; Pitt and Stanley; 2014; Nolff et al., 2015; Howe et al., 2015). However, applying a NPWT bandage in an animal, and especially in horses, brings along certain difficulties. A horse's skin for example is covered with hairs and is much greasier compared to human skin. Additionally, horses cannot be told to immobilize a certain part of their body. Therefore, a guideline is presented on how to apply a NPWT bandage to a horse with practical tips based on the personal experience of the author. The guideline is based on the application of the commercial V.A.C. product line of KCI medical Inc., since this is the system currently used in our institution.

2 HOW TO APPLY NEGATIVE PRESSURE WOUND THERAPY ON A HORSE

Wound preparation

Following consecutive steps should to be taken 24 hours before application of a NPWT bandage:

1. Clip the hairs surrounding the wound
2. Shave the clipped area with a razor blade (Fig. 11)
3. Debride the wound

Figure 11. Preparation of a wound for NPWT.
The hairs adjacent to the wound have to be removed before applying negative pressure wound therapy. The hairs are first clipped and then shaved with a razor blade.

Application of the NPWT bandage

A NPWT bandage can be applied in a horse using the following protocol:

1. Clean the wound thoroughly
2. Degrease and clean the surrounding shaved skin with ether (protect the wound with a clean and sterile gauze)
3. Apply adhesive spray to the surrounding skin
4. Remove the protective gauze from the wound and cut the foam to the size of the wound
5. Cut the adhesive occlusive drape in strips to facilitate application
6. Insert the foam into the wound, ensure all cavities and underminings are filled
7. Secure the foam into wound using a small strip of the occlusive drape (Fig 12)

8. Put the canister in the NPWT system
9. Cover the foam enterily by imbricating the strips of occlusive drape. Make sure the drape overlaps minimally 6 cm onto the surrounding skin.
10. Cut a circular opening of approximately 2 cm into the drape covering the foam (Fig. 13)
11. Apply the evacuation tube over the opening, ensure the tube runs proximally/ dorsally (Fig. 13)

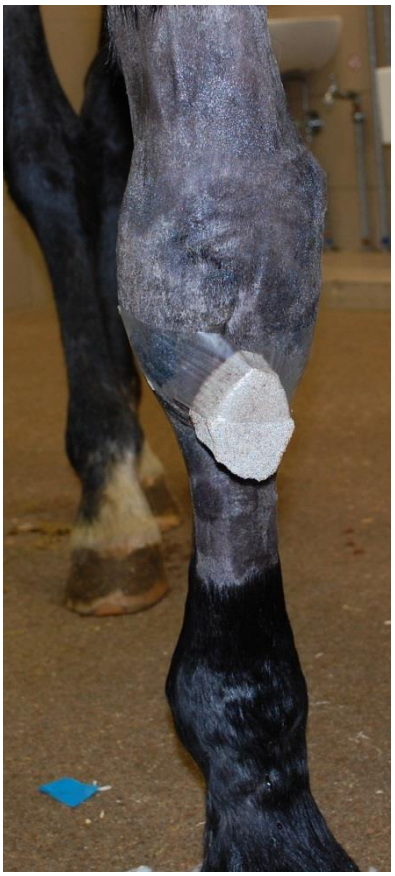


Figure 12. Application of a negative pressure wound therapy bandage.
The foam can be secured into the wound by a small strip of adhesive occlusive drape before the entire wound area is covered with adhesive occlusive drape.

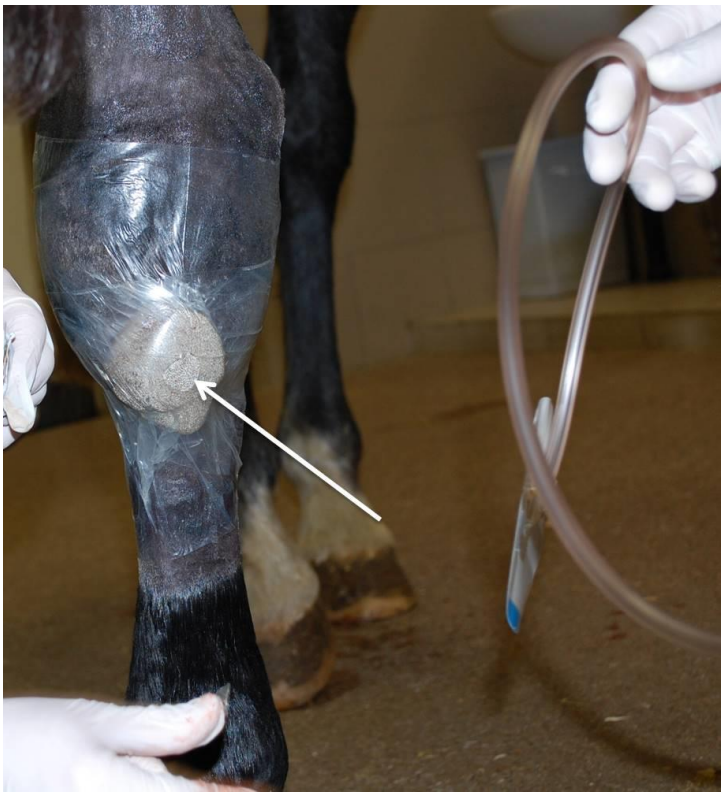


Figure 13. Application of a negative wound therapy bandage.
A 2cm circular opening (indicated by the white arrow) is cut into the adhesive occlusive drape covering the foam. On this circular opening the evacuation tube will be applied

12. Connect the evacuation tube with the tube of the canister in the NPWT system. Make sure the clamps are open
13. Choose the preferred pressure (commonly -125 mm Hg) and suction mode (commonly continuous)
14. Check on the screen if the pressure builds up and check if the foam collapses (Fig. 14). If necessary, apply light pressure with your hands to press the air out of the foam to help it collapse. If leaks are present, apply extra strips of drape.
15. If the wound is on an anatomically challenging location (e.g. a place with a lot of movement), an elastic adhesive bandage can be applied at the edges of the adhesive occlusive drape to secure it extra to the skin
16. Apply a standard limb bandage if the NPWT is on a limb.
17. Immobilize the horse by tethering it in a small stable (Fig. 15) or use a mobile NPWT system.

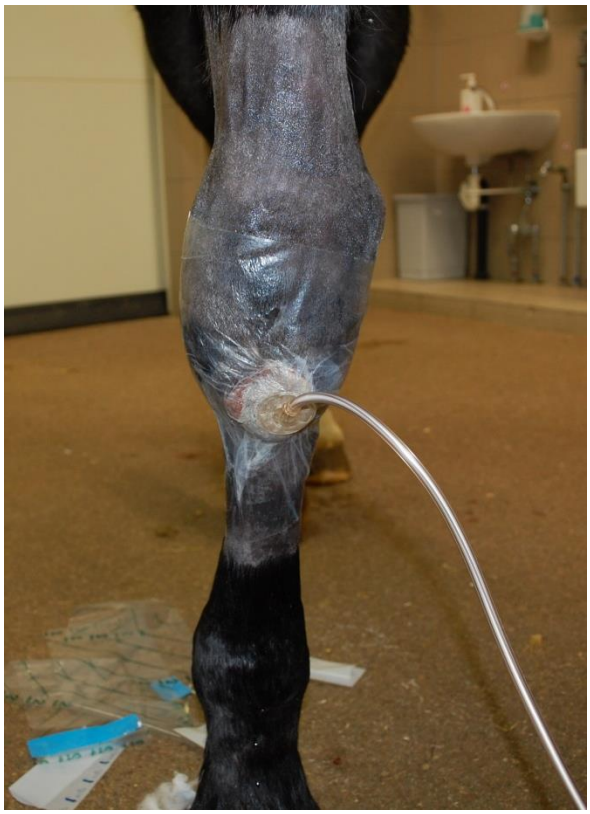


Figure 14. A NPWT bandage applied on an infected precarpal bursa in a horse. When the negative pressure is delivered to the foam, the foam collapses and excess wound fluid is removed from the wound bed.

Figure 15. A horse with a negative pressure wound therapy bandage applied to the right front limb. The horse is placed in narrow stable and tethered at both sides of the head to minimize the mobility of the horse. The animal should not get stuck in the tubes of the negative pressure wound therapy bandage and should not pull the negative pressure system off the wall.

Tips and considerations when applying a NPWT bandage to a horse

- In horses, the hairs adjacent to the wound have to be removed in order for the adhesive occlusive drape to stick on the skin. The shaved area should extend at least ten cm from the wound edges. To the author's experience, the adhesive drape does not stick sufficiently if the hairs are only clipped, so the skin also has to be shaved with a razor blade. Hairs have to be removed the day before application of the bandage, since shaving with a razor blade can give little nicks and cuts. Consequently, blood from these cuts can get beneath the adhesive occlusive drape and prevent an airtight seal.
- Since the effect of NPWT on bacterial load is still controversial, a wound should be thoroughly cleaned and debrided before application a NPWT bandage (Mouës et al., 2011, Patmo et al., 2014). The debridement has to be performed at the latest 24 hours before application of the bandage, because the wound should not be bleeding anymore when applying NPWT.
- In horses, it is important to degrease the skin with ether, because their skin is oilier than in humans. Together with the application of adhesive spray on the skin, this improves the seal between the adhesive occlusive drape and the skin.
- When handling the adhesive occlusive drape and the wound filler (the foam), it is recommended to wear sterile gloves or at least new clean non-sterile gloves to minimize contamination. For the same reason the cutting of the drape and wound filler is best performed with a sterile scissor.
- Between step five and six, the following additional steps can be taken to prevent the foam from overlapping onto the surrounding skin and thus irritating it.
    - Cover the skin and wound with the adhesive occlusive drape
    - Cut the wound free, make sure the adjacent skin stays covered with the occlusive drape
- To facilitate cutting the foam to the size of the wound, an impression of the wound can be made on the foam. To do this the foam is pressed gently against the wound bed. The excess foam can then be cut off. However, it is best to leave a margin of approximately 1 cm, since the foam collapses and thus shrinks a bit when the negative pressure is applied.
- The presence of undermined regions in a wound can be measured with the aid of a sterile cotton swab.
- When inserting the wound filler into the wound and underminings, make sure the foam is not completely squeezed together, since this will prevent the foam to efficiently deliver the negative pressure equally to the entire wound surface.

- If the wound is very exudative, a clean sterile gauze can be placed distally to the wound to absorb excess wound fluid after inserting the wound filler. This excess wound fluid will otherwise prevent the adhesive occlusive drape from sticking to the skin. This gauze can be left in place until the last piece of adhesive drape is applied to create the airtight seal of the wound. After removing the gauze, the negative pressure has to be established as soon as possible to prevent the wound fluid from running between the drape and skin thus breaking the seal.
- To cut the opening in the adhesive occlusive foil for the evacuation tube, a sterile forceps can be used to lift the drape from the foam.
- The limb bandage preferable consists of cotton wool as a second layer and an elastic bandage as third layer and helps to maintain the integrity of the NPWT bandage.
- To prevent the development of pressure wounds from the tubing, a separate piece of cotton wool or padding has to be applied between the skin and the tube.
- The tubing can additionally be secured proximally on the limb by using an adhesive elastic bandage as a first layer onto which the tube can be fixed with tape.
- The negative pressure system is best monitored every 1 to 2 hours for leaks, blockades and other malfunctions.
- If the NPWT bandage does not function anymore, it has to be replaced or removed, but should never left be in place as this stimulates maceration of the skin and development of wound infections.
- If the horse has to be tethered it is best to mildly sedate it using acepromazine orally and ensure the horse has ad libitum access to roughage.

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SCIENTIFIC AIMS

Negative pressure wound therapy (NPWT) is being used successfully in human medicine to treat a wide variety of wounds and it could aid to overcome typical problems seen with second intention wound healing at the equine distal limb. However, studies on NPWT in horses are lacking. Therefore, the **general aim** of this PhD study was to assess the influence of negative pressure wound therapy (NPWT) on second intention wound healing in the equine distal limb.

To assess second intention wound healing, several parameters have to be evaluated such as wound dimensions and bacterial load. Therefore, the **first objective** of this PhD study was to compare a new laser beam wound camera to a digital photoplanimetry-based method for wound measurement in horses. The results of this study were used to select the wound measurement technique for the further *in vivo* studies. The **second objective** was to compare different methods to evaluate bacterial load in second intention healing wounds and to assess the impact of a biofilm-disrupting protocol on the bacterial counts of equine wound samples with histologically observed biofilms. The results of this study were used to select the *modus operandi* to monitor the bacterial load in the further *in vivo* studies.

The influence of NPWT on bacterial load is still controversial, even in human medicine where this technique is used frequently. Research on this subject displays a great variety in study designs with consequently variable results. Moreover, a comparison of the antibacterial effect of different wound fillers used with NPWT is lacking in the literature. Therefore, the **third objective** of this PhD study was to evaluate the antibacterial effect of three different wound fillers used with NPWT in an *ex vivo* model. The results of this study were used to select the most appropriate wound filler for the further *in vivo* studies.

The **fourth objective** of this PhD study was to assess the clinical relevance of NPWT for the treatment of acute and contaminated second intention healing wounds on the equine distal limb. Therefore, an *in vivo* experimental wound model was developed and appraised, through which the effect of NPWT on the inflammatory response, wound dimensions, bacterial load, blood flow, angiogenesis and the presence of myofibroblasts could be evaluated in both acute non-inoculated and inoculated second intention healing distal limb wounds.

COMPARISON OF A NEW LASER BEAM WOUND CAMERA AND A DIGITAL PHOTOPLANIMETRY-BASED METHOD FOR WOUND MEASUREMENT IN HORSES

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Summary

The aim of this study was to compare the accuracy, precision, inter- and intra-operator reliability of a new laser beam (LB) wound camera and a digital photoplanimetry-based (DPB) method for measuring the dimensions of equine wounds. Forty-one wounds were created on equine cadavers. The area, circumference, maximum depth and volume of each wound were measured four times with both techniques by two operators. A silicone cast was made of each wound and served as the reference standard to measure the wound dimensions.

The DPB method had a higher accuracy and precision in determining the wound volume compared with the LB camera, which had a higher accuracy in determining the wound area and maximum depth and better precision in determining the area and circumference. The LB camera also had a significantly higher overall inter-operator reliability for measuring the wound area, circumference and volume. In contrast, the DPB method had poor intra-operator reliability for the wound circumference. The LB camera was more user-friendly than the DPB method.

The LB wound camera is recommended as the better objective method to assess the dimensions of wounds in horses, despite its poorer performance for the measurement of wound volume. However, if the wound measurements are performed by one operator on cadavers or animals under general anaesthesia, the DPB method is a less expensive and valid alternative.

Introduction

Traumatic wounds are very common in horses and are often left to heal by second intention because of massive tissue loss, heavy bacterial contamination, or high skin tension (Wilmink et al., 2002; Theoret, 2008a). Second intention wound healing is often delayed due to infection, chronic inflammation, the presence of foreign bodies or impaired blood supply, so the choice of topical treatment is very important and should be based on careful monitoring of wound healing. Besides a subjective evaluation of the degree of inflammation and the quality and quantity of the granulation tissue, an objective and correct measurement of the wound dimensions (area and depth) is essential to follow the progress of wound healing through contraction and epithelisation. Objective monitoring allows for more rapid intervention and adaptation of the treatment plan when the wound deteriorates (Sibbald et al., 2006; Theoret, 2008b; Wilmink, 2008). Furthermore, objective evaluation of wound dimensions can facilitate clear communication to the horse owner about the progress of healing, and can aid high quality research designed to investigate wound healing.

Granulation tissue formation plays a crucial role in equine wound healing in the horse (Theoret, 2008a) but its formation cannot be monitored by area and circumference measurements only, especially in the early stages. During this early period, granulation tissue starts to form at the base of the wound, but the wound surface area remains unchanged (Little et al., 2009), so depth and volume measurements are essential to monitor progress. Area measurement of wounds is difficult when the skin surface is curved, or if the digital camera is not perpendicular to the wound surface (Treuillet et al., 2009). Therefore, it is important in measuring wounds to use a three dimensional technique, such as structured light or stereophotogrammetry (which can compensate for the skin curvature). Measuring wound dimensions in horses is more challenging than measuring wounds in humans and the biggest problem is immobilisation of the animal during the procedure. A measurement technique that requires contact with the wound is often difficult to perform accurately and is likely to be time-consuming, especially when the wound is painful. Moreover, contact with the wound increases the risk of contamination and so a fast non-contact technique is preferable. The digital photoplanimetry (DP) technique, commonly used in equine studies to evaluate wound dimensions (Berry and Sullins, 2003; Monteiro et al., 2009; Tóth et al., 2011; Azari et al., 2012; Bischofberger et al., 2013), meets the noncontact requirement, but cannot measure either the depth or volume of a wound. Moreover, the accuracy or precision of the technique is unknown, reflecting a dearth of published information on wound measurement techniques in veterinary medicine. Only one study has been published on the feasibility and intra-operator variability of stereophotogrammetry (Labens and Blikslager, 2013), and to our

knowledge, research investigating the accuracy and precision of other wound measurement techniques has not been published.

The aim of the present study was to compare two wound measurement techniques in horses. DP was chosen as the first technique, since it is frequently used in equine studies to assess wound dimensions (Berry and Sullins, 2003; Monteiro et al., 2009; Tóth et al., 2011; Azari et al., 2012; Bischofberger et al., 2013). The technique was supplemented with a manual measurement of the maximum wound depth, thus allowing the wound volume to be calculated using the validated Kundin formula (Kundin, 1989; Langemo et al., 1998, 2001). For the second technique, a new laser beam (LB) wound camera (SilhouetteStar, ARANZ Medical) was chosen. The accuracy, precision, inter- and intra-operator reliability of the two techniques for the measurement of equine wounds were assessed. Silicone wound casts served as the reference standard to measure wound dimensions.

Materials and Methods

Forty-one wounds were created on equine cadavers collected from the Pathology Department of the Faculty of Veterinary Medicine, Ghent University. The cadavers originated from horses euthanized for reasons other than the conduct of this study and informed consent was obtained from the owners of the horses used. If the horses were euthanized more than 2 h before creating the wounds, their cadavers were stored in a refrigerator at 4 °C until the start of the experiment. After clipping, wounds were created with a scalpel on either the neck, or the thorax, or the limbs proximal to the carpus or tarsus, by removing the skin and a piece of the underlying muscle depending on the desired depth of the wound. The wounds were created 1–48 h after euthanasia and varied in surface, depth, shape and size to resemble real-life clinical cases.

For each wound, the surface area, circumference, maximum depth and volume were measured. All wounds were measured with the DP-based (DPB) method first, followed by the LB camera by two different operators to determine inter-operator reliability; one operator had experience with wound management, whereas the other did not. Each wound was measured four times with each technique to assess the intra-operator reliability. After performing the wound measurements, silicone casts were made of the wounds to serve as the reference standard. During the experiment, the cadavers were not moved so that distortion of the soft tissues that could lead to a change in the wound dimensions was avoided.

Digital photoplanimetry-based method

The wounds were photographed with a digital camera held perpendicular to the

wound surface. A ruler positioned next to the wound served as a metric scale (Fig. 1). To measure the maximum depth, a cotton-tipped swab was positioned in the deepest part of the wound and was pinched between thumb and index finger at the level of the surrounding skin. The swab was then removed from the wound while remaining pinched between thumb and index finger, before the distance from the cotton tip to the top of the fingers was measured with a ruler. The pictures were analysed with an open source Java-based program to calculate the surface area and the circumference of the wound.[1]

The volume of the wound was calculated using the Kundin formula:

Volume = Area x maximum depth x 0.327

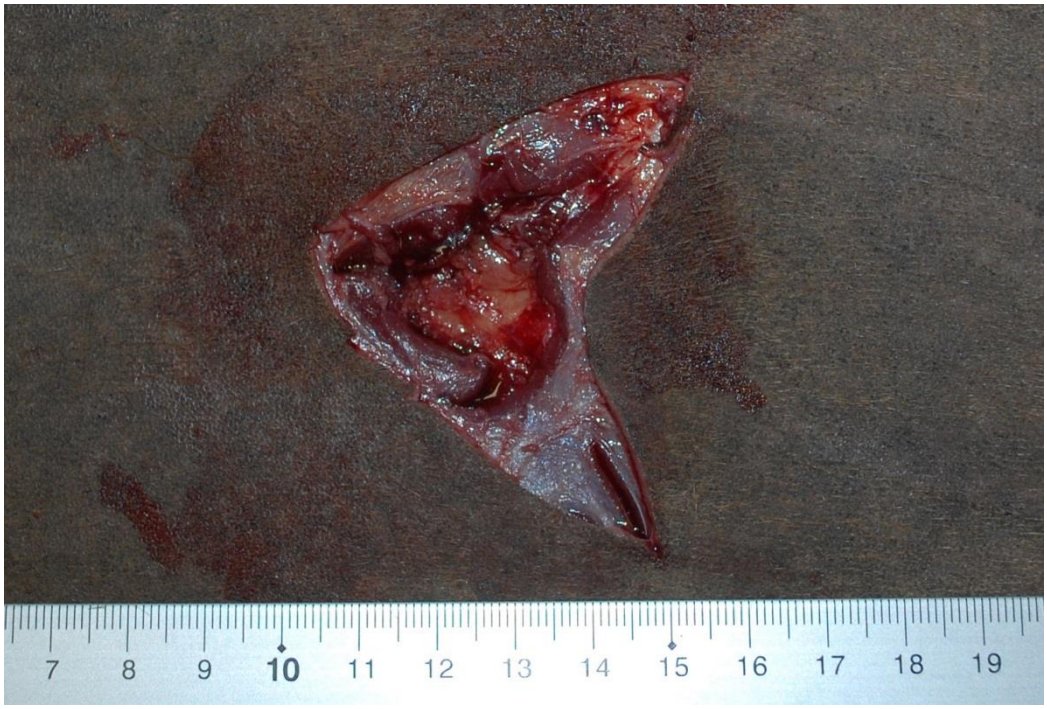

Figure 1. Picture obtained perpendicular to the wound surface with a metric scale marker included for analysis within the ImageJ[1] program for the digital photoplanimetry-based method.

Laser beam wound camera

First, the LB camera (SilhouetteStar, ARANZ Medical) was connected to a laptop containing the appropriate software (SilhouetteConnect, ARANZ Medical). The camera was held perpendicular to the wound surface as described in the manufacturer's instructions. Ideally, the three laser lines projected by the camera on the wound surface crossed in a star shape,

[1] See: Rasband, W.S., ImageJ, U. S. National Institutes of Health, http://imagej.nih.gov/ij/ (accessed 22 June 2016).

with the ends of the lines lying on the adjacent skin and the central focus point of the star at or near to the centre of the wound (Fig. 2). The operators ensured that at least a part of one of the laser lines always ran over the deepest part of the wound. After taking a picture, the circumference, maximum depth and volume of the wound were calculated using the accompanying software.

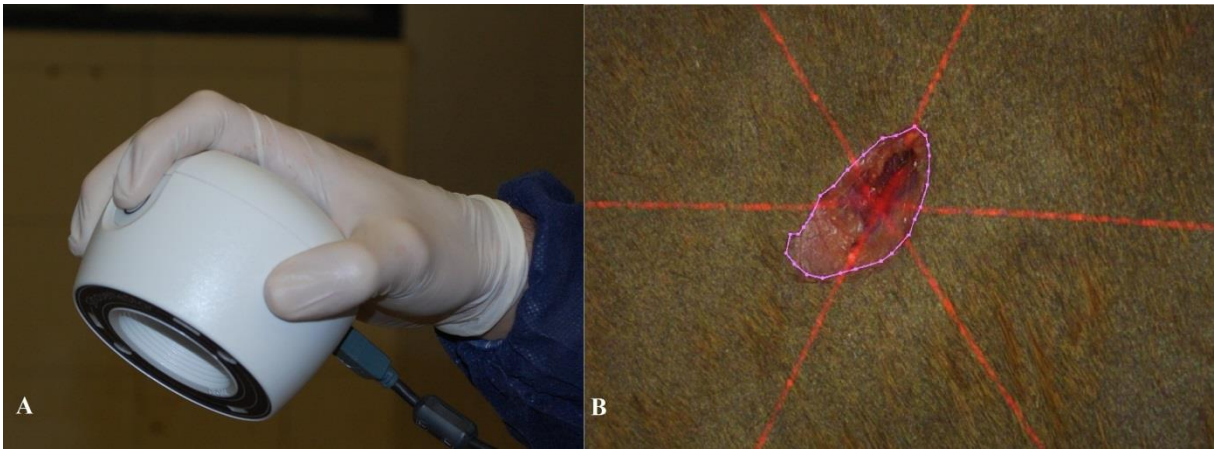

Figure 2. (A) The laser beam wound camera and (B) image of a wound captured with the laser beam wound camera.

The three laser lines projected by the camera on the wound surface form a star shape with the lines crossing at or near the centre of the wound with the ends of the lines lying on the adjacent skin.

The software used the three laser lines projected on the wound surface as a ‘scaffold’ to build a mathematical model of the wound (Fig. 3a). A mathematical surface was drawn over the laser lines as shown in Fig. 3b, onto which the software added the wound boundaries as traced by the operator (Fig. 3c). For concave wounds (as was the case for all the wounds in our study), the software stretched a virtual cap over the mathematical wound concavity, which represented a reconstruction of the former intact skin and served as a digital reference to calculate the maximum depth and volume of the wound (Figs 3d and 4).

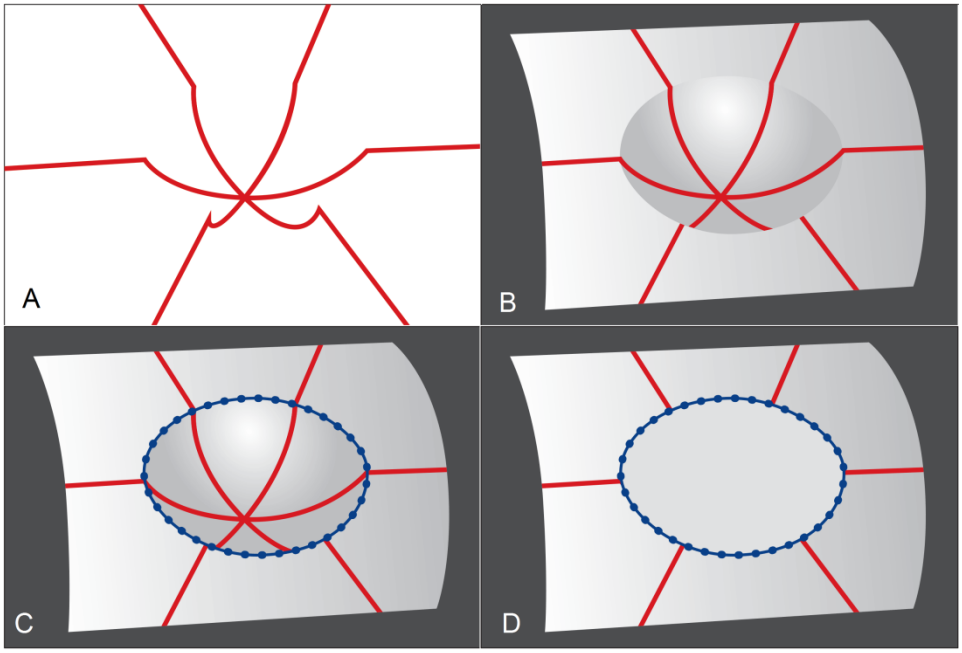

Figure 3. Schematic presentation of the working mechanism of the SilhouetteConnect software. (A) The three laser lines projected by the laser beam wound camera on the wound surface are drawn in a three-dimensional space. (B) A mathematical surface is drawn over the three laser lines to build a model of the wound. (C) The wound boundary as traced by the operator is added. (D) The software stretches a virtual cap over the wound concavity to reconstruct the missing skin.

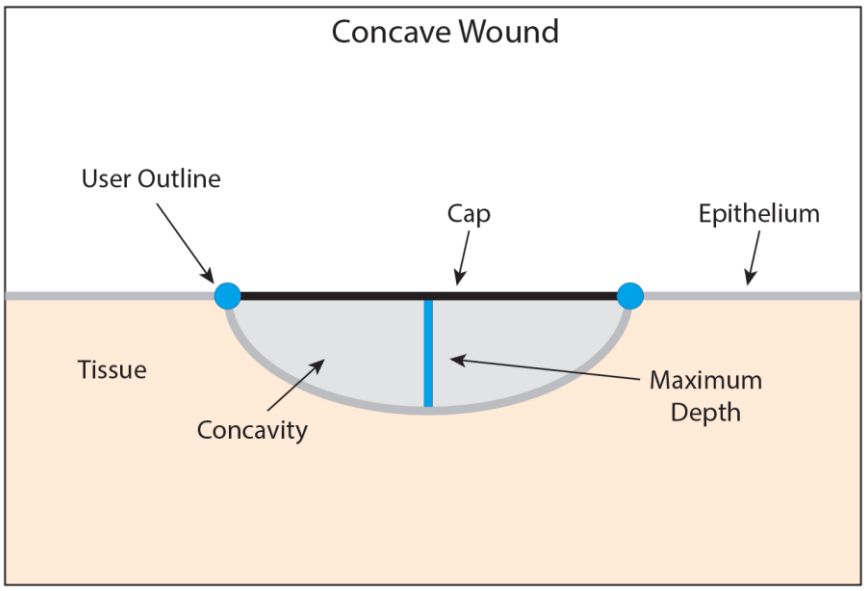

Figure 4. The SilhouetteConnect software calculates the maximum depth and volume of a wound based on a virtual cap stretched over the wound defect, which represents the former intact skin.

Silicone wound casts

Silicone wound casts were used as the reference standard to measure wound dimensions. The wound casts were made after the measurements with the DPB method and the LB camera were completed, since removing the casts could change the wound dimensions. For the same reason, only one cast was made of each wound. For all casts, body double 'fast set' silicone (FormX) was used, because silicone does not shrink and provides highly detailed reproductions. The wounds were first covered with transparent plastic to prevent the silicone from extending above the level of the surrounding skin. Next, the silicone was injected beneath the plastic foil into the wounds with aid of a dispensing gun to minimise spillage and to obtain an optimal mix of the silicone components. The casts were removed from the wound bed after 5–15 min, when the silicone was set.

To measure the area and circumference of a cast, it was photographed with a digital camera held perpendicular to its surface. A ruler positioned next to the cast served as a metric scale (Fig. 5). ImageJ[1] was used to calculate the area and circumference of the cast surface. To calculate the maximum depth of a cast, it was photographed with a digital camera held perpendicular to the side of the cast (Fig. 6). A ruler held next to the cast served as a metric scale. The pictures were also analysed with ImageJ[1] to calculate the maximum depth of the wound cast. To measure the volume of a wound cast, the cast was weighed and the mass was divided by the specific gravity of the silicone (1.17 g/cm^3). All cast dimensions measurements were repeated four times by the same operator. The values of the cast dimensions represented the true wound dimensions.

Figure 5. Picture perpendicular to the surface of a wound silicone cast with a metric scale to be processed with the ImageJ[1] program to calculate the area and circumference of the cast.

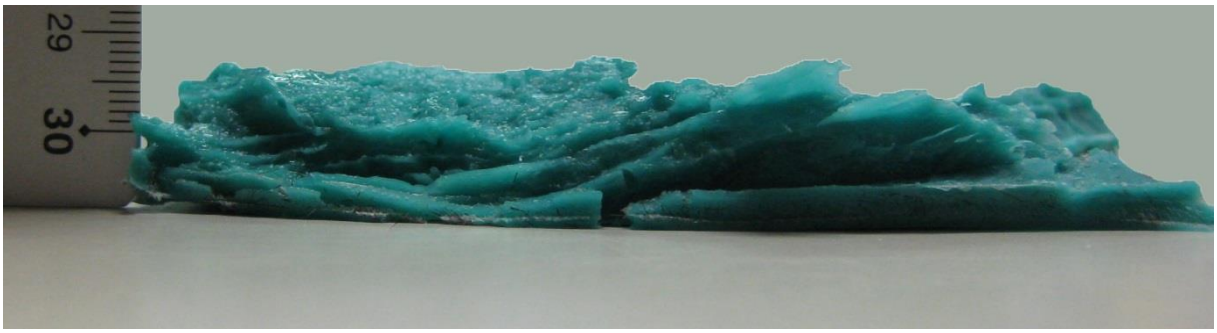

Figure 6. Picture perpendicular to the side of the silicone cast with a metric scale to be processed with the ImageJ[1] program to calculate the maximum depth of the cast.

Data processing

Since the true wound dimensions were measured, the relative bias (RB) of the DPB method and the LB camera could be calculated for each wound (Langemo et al., 1998, 2001). The RB is a measure of the accuracy of a technique. In this study, the average value of wound dimensions across operators and repeated measurements was used to calculate RB. The following equation was used to calculate RB for the two techniques (Langemo et al., 2001):

$$RB = \left(\frac{[\text{mean wound dimension technique} - \text{true wound dimension}]}{\text{true wound dimension}}\right) * 100$$

RB was interpreted by its absolute value; low RB values indicated an accurate technique.

The precision of the technique was assessed by calculating the standard error of the measurement (SEM; Langemo et al., 1998, 2001). The SEM for each technique was calculated as follows (Langemo et al., 2001):

$$\text{SEM} = \sqrt{\frac{\sum_{i=1}^{n}(\text{wound dimension technique} - \text{true wound dimension})^2}{n}}$$

The average value of the wound dimension across operators and repeated measurements was used in this calculation. The SEM was interpreted by its magnitude; low SEM values indicated a precise technique.

Statistical analysis

Two-tailed paired Student's t tests were performed to determine whether the mean RB for wound dimensions differed significantly between the DPB method and the LB camera. The normality of the differences was verified with Q-Q plots. For the wound circumference, the measurement differences were not normally distributed, so Wilcoxon signed-ranks tests were used to determine if there were significant differences between the two techniques. P values of <0.05 were regarded as statistically significant for all tests.

The inter-operator reliability of the two techniques was determined by an intraclass-correlation (ICC) test for absolute agreement in a two-way random model, where the ICC test value ρI = 0 represents no reproducibility and ρI = 1 represents perfect reproducibility between operators. The interpretation of ρI was based on the guidelines by Rosner (2011): ρI < 0.4 was regarded as poor reproducibility, while 0.4 ≤ ρI < 0.75 or ρI ≥ 0.75 was regarded as a fair to good or excellent reproducibility, respectively. The ICC test values were considered significantly different between the DPB method and the LB camera if the 95% confidence intervals of the values of interest did not overlap.

The intra-operator reliability of the two techniques was determined by a multivariate analysis of variance (MANOVA) for repeated measures. P values < 0.05 were regarded as statistically significant. All statistical analyses were performed using IBM SPSS version 21.0 (IBM).

Results

Accuracy

An overview of the mean RB for the different wound dimensions per wound measurement technique is shown in table 1. The mean RB for the wound area and maximum depth were significantly smaller when the LB camera was used compared with the DPB method (P <

0.001). In contrast, the DPB method had a significantly smaller mean RB for wound volume ($P < 0.001$). The mean RB between the two techniques did not differ significantly for wound circumference ($P > 0.05$). The LB camera underestimated all the wound dimensions, whereas the DPB method underestimated the wound area, circumference and volume, but overestimated the maximum wound depth.

Table 1. Accuracy of the digital photoplanimetry-based method and the laser beam wound camera for the different wound dimensions.

	Digital photoplanimetry		Laser beam wound camera	
	Mean RB[a]	SD	Mean RB[a]	SD
Area	-12.4 %[b]	8.3	-8.6 %[b]	6.3
Circumference	-1.1 %	8.4	-2.9 %	7.0
Maximum depth	53.5 %[b]	52.9	-7.4 %[b]	39.5
Volume	-10.2 %[b]	33.0	-44.1[b]	26.1

RB, relative bias; SD, standard deviation

[a] Mean relative bias (RB) indicates whether the technique overestimates (+) or underestimates (-) the wound dimension compared to the reference standard. The smaller the absolute value of the RB, the more accurate the technique.

[b] Values differ significantly ($P<0.01$) when compared to the other technique for the same wound dimension

Precision

The SEMs for the different wound dimensions for each technique are shown in table 2. For area and circumference, the LB camera measurements had smaller SEMs, which indicated that it was more precise compared with the DPB method. The SEM for maximum wound depth was the same for the DPB method and the LB camera. The SEM for volume was smaller for the DPB method compared with the LB camera.

Table 2. The standard error of measurement (SEM[a]) of digital photoplanimetry-based method and the laser beam wound camera for the different wound dimensions.

	Digital photoplanimetry	Laser beam wound camera
Area	10.53	6.4
Circumference	2.43	1.83
Maximum depth	0.61	0.61
Volume	10.63	17.06

[a] The standard error of measurement (SEM) indicates the spread of the error in measurements; lower SEM indicates a more precise technique.

Inter-operator reliability

table 3 shows the results for the intra-class correlation test (ICC) for each measurement technique. The values are displayed for single measures and average measures. The ICC values were significantly higher for the LB camera compared with the DPB method for wound area, circumference and volume. For the maximum depth, the ICC values of the camera tended to be higher, but the difference was not statistically significant. Nonetheless, the ICC values for both techniques indicated that reproducibility between operators was excellent.

Table 3. Inter-operator reliability (intra-class correlation, ICC; ρ_I) for digital photoplanimetry-based method and the laser beam wound camera for all wound dimensions [a]

	Single measures [b]	Average measures [c]
Digital photoplanimetry		
Area	0.997[d]	0.999[d]
Circumference	0.987[d]	0.994[d]
Maximum depth	0.923	0.960
Volume	0.950[d]	0.974[d]
Laser beam wound camera		
Area	0.999[d]	1.000[d]
Circumference	0.993[d]	0.996[d]
Maximum depth	0.946	0.972
Volume	0.978[d]	0.989[d]

[a] Larger ICC test values indicate a better reproducibility between operators.
[b] The values for the single measures represent the agreement between operators for a single measurement and are important when the wound measurement technique will be performed by one operator, which is often the case in research.
[c] The values for the average measures represent the agreement between operators on average and are important when the wound measurement technique is performed by several operators, which is predominantly the case in a clinical setting.
[d] ICC test values differ significantly ($P < 0.05$) when compared to the other technique for the same wound dimension.

Neither technique showed reproducibility (ρI did not differ significantly from 0) for the maximum depth of superficial large wounds, so inter-operator agreement was poor. Nonetheless, for the other wounds and dimensions, reproducibility for both the LB camera and the DPB method was excellent.

Intra-operator reliability

There were no significant differences between the repeated measurements for any of the wound dimensions for the LB camera, indicating good intra-operator reliability. However, for the DPB method, a significant difference was found between the repeated measurements of one operator for wound circumference ($P = 0.003$), more specifically for superficial large wounds ($P = 0.011$). Therefore, for wound circumference, the DPB method had poor intra-operator reliability. For maximum wound depth, the DPB method was repeatable for the wound groups collectively, but not for deep small wounds ($P = 0.016$).

Discussion

The goal of this study was to compare a DPB method and a new LB camera for the objective measurement of wound measurement in horses. Accuracy, precision, inter- and intra-operator reliability data for these two techniques demonstrated that the LB camera was the better method because of its higher accuracy (smaller mean RB) for the determination of wound area and maximum depth and its better precision (smaller SEM) for the determination of wound area and circumference. Although the DPB method sometimes had higher accuracy for the measurement of wound circumference, its precision was lower compared with the LB camera. The DPB method also had poor intra-operator reliability for wound circumference.

Even though the wound volume was more accurately measured with the DPB method (as mean RB and SEM was less than for the laser camera), the LB camera had a better inter-operator reliability for measurement of wound volume. Furthermore, a manual depth measurement was necessary to calculate the volume of the wound when DP was used. This manual depth measurement required contact with the wound, which could cause pain and aversive behaviour in live horses, thereby preventing accurate measurement. In particular, when a wound is painful, adequate restraint to obtain accurate readings of maximum wound depth could be difficult, resulting in greater variability in wound volume measurement under field conditions.

The LB camera was found to be a more user-friendly system compared with the DPB method. Capturing the picture, assigning the correct label in the software and tracing the wound boundaries required about 2–5 min. With the DPB method, a manual depth measurement had to be performed after taking the picture, after which the pictures had to be transferred to a computer for image processing. Therefore, the total time necessary to capture a picture, perform the manual depth measurement, transfer the pictures to a computer, trace the wound boundaries and calculate the volume of the wound with aid of the Kundin formula required 10–15 min. Regardless of these differences, both techniques can be performed in field settings.

The LB camera is relatively expensive in comparison with traditional wound measurement equipment and this might make the use of the system impractical in veterinary field practice. Nevertheless, the LB camera is well suited to clinical settings where there are high caseloads of complicated and difficult wounds that might be slow to heal.

Objective measurements of wound dimensions are important to ensure adequate monitoring of wound healing and to enable unambiguous communication with horse owners. Objective

measurements also facilitate good documentation and standardised measurements for research. Based on the results of this study, the LB camera can be recommended for objective wound measurement by several different operators, since it has better inter-operator reliability than the DPB method. However, if wound measurements are performed by one operator on cadavers or animals under general anaesthesia, digital photoplanimetry is less expensive and a valid alternative.

We decided to use an ex vivo wound model to validate the measurement techniques. By using an ex vivo wound model, the variability in discerning and tracing wound boundaries was taken into account when testing the accuracy and precision of the wound measurement techniques. Moreover, changes in wound dimensions between repeated measurements, or measurement methods when the horse changes position or flexes or relaxes a muscle, were avoided with this model. The ex vivo wound model also made it possible to create a wide range of wounds and to use silicone wound casts as the reference standard. Nevertheless, a perfect negative cast was difficult to obtain, since the reconstruction of the exact curvature of the missing skin was difficult. A plastic cover was used to prevent the silicone material from exceeding the level of the surrounding skin. Even so, light bulging of the silicone material over the skin level was sometimes observed, which mainly impacted depth and volume measurements in superficial wounds. However, the variation in wound dimensions using the casts was very small compared with measurements made using the LB camera and the DPB method.

One limitation of this study was the exclusion of wounds that were too large to be included completely in one photograph (e.g. circumferential wounds on distal limbs). However, adaptations of the LB camera or the DPB method could be developed to measure large wounds. To address this problem, multiple pictures should be taken, using markers to serve as reference points to divide the wound into two or more parts. With the DPB method used in this study, it would be possible to measure the area, circumference, maximum depth and volume of such wounds. The LB camera can combine two or more pictures to measure the area and circumference of such wounds, but maximum depth and volume cannot be calculated with currently available software. The accuracy and precision of these techniques for these types of wounds should be investigated.

Conclusions

The DPB method had a higher accuracy and precision for the determination of wound volume, whereas the LB camera had higher accuracy in determining wound area and maximum depth and superior precision for the determination of wound area and circumference. Compared with the DPB method, the LB camera also had a significantly

higher inter-operator reliability for measuring wound area, circumference and volume. In contrast, the DPB method had poor intra-operator reliability for wound circumference measurement. In addition, the LB camera was more user-friendly compared with the DPB method. Based on the results presented here, we recommend the LB camera should be used for the objective assessment of the dimensions of wounds in horses, despite its inferior performance in measuring wound volume. However, if wound measurements are performed by one operator on cadavers or animals under general anaesthesia, the DPB method is an inexpensive and valid alternative.

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A COMPARISON OF DIFFERENT METHODS TO DETERMINE BACTERIAL LOAD IN SECOND INTENTION HEALING WOUNDS IN HORSES

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Summary

The aim of this study was to evaluate different techniques to diagnose infection in second intention healing wounds in horses and to assess the influence of a vortex and sonication protocol on quantitative bacteriology in equine wound samples with a histologically confirmed biofilm. In fifty second intention healing wounds, a clinical assessment, quantitative swab, semi-quantitative swab, and a swab for cytology were compared to a quantitative tissue biopsy (reference standard). Part of the biopsy was examined histologically for evidence of a biofilm.

A high correlation ($P < 0.001$ $r = 0.747$) was shown between the outcome of the quantitative swabs and the quantitative biopsies. These were linearly related ($P < 0.001$) by the regression function $Y = 1.121 + 0.846\ X$ ($r^2 = 0.56$), where Y represents the log colony-forming units (CFU) of the quantitative biopsy and X represents the log CFU of the quantitative swabs. The semi-quantitative swabs showed a significant but only moderate correlation with the quantitative biopsies ($P < 0.001$ $\rho = 0.524$). Higher white blood cell counts for cytology were significantly associated with lower log CFU in the wounds ($P = 0.02$). Wounds with black granulation tissue showed significantly higher log CFU ($P = 0.003$). Samples with biofilms did not give a higher bacteriological count after a vortex and sonication protocol was performed to release bacteria from the biofilm. Based on the findings in this study, the quantitative swab seems a good non-invasive alternative to the quantitative biopsy to quantify bacterial load in equine wounds.

Introduction

Traumatic wounds in horses are often a cause of noteworthy morbidity and mortality (Theoret et al., 2016). Additionally, traumatic wounds are always contaminated with micro-organisms (Provost, 2012). This contamination can evolve towards an infection depending on several factors, such as the virulence of the micro-organisms, the number of micro-organisms, the nature of the wound, and the host's immune response (Sibbald et al., 2006). Since infection has a detrimental effect on wound healing, an early detection is of utmost importance (Stashak, 2008a).

In human medicine, the golden standard to diagnose wound infection is a quantitative tissue biopsy (Robson, 1997; Ratliff and Rodeheaver, 2002; Dow, 2003; Serena et al. 2006; Bonham, 2009). The presence of more than 10^5 colony-forming units (CFU) per gram of tissue is generally accepted to indicate infection (Robson, 1997; Bowler et al., 2001; Rondas, 2013). The validity of this 10^5 guideline has been confirmed in horses in the past (Peyton and Connelly, 1983). However, in human medicine several studies have demonstrated more practical and less invasive alternatives for a tissue biopsy to quantify bacterial load, whilst in veterinary medicine these alternatives have not been evaluated (Levine et al., 1976; Bowler et al., 2001; Gardner et al., 2001; Ratliff and Rodeheaver, 2002; Gardner et al. 2006; Serena et al., 2006; Davies et al., 2007; Woo and Sibbald, 2009).

In equine medicine, the 10^5 guideline is recommended to diagnose infection in traumatic wounds in combination with qualitative bacteriology (Hendrickson and Virgin, 2005; Stashak, 2008b, Provost, 2012). Nevertheless, wound infection is generally diagnosed based purely on clinical signs in equine practice. However, to the authors' knowledge, studies investigating which clinical signs indicate a high bacterial load are lacking in horses.

The significance of the presence of biofilms in equine wounds is increasingly recognised as a key factor in wound infections (Freeman et al., 2009). Biofilms are thought to delay wound healing, because the bacteria in the biofilms have enhanced virulence and are protected from the immune response of the host. Moreover, bacteria in biofilms are more resistant to antimicrobials (Freeman et al, 2009). In human wounds, the presence of biofilms has been increasingly investigated the last decade (Serralta et al., 2001; Percival and Bowler, 2004; Davis et al., 2008; Mancl et al., 2013). Biofilms have also been demonstrated in equine wounds (Freeman et al., 2009; Westgate et al., 2011). Bacteria in these biofilms are embedded in an extracellular polymeric substance in the form of microclusters and are in a slow-growing or non-growing state. Therefore, a number of these bacteria are potentially missed during qualitative and quantitative bacteriological analysis. However, to our

knowledge, research on the impact of a biofilm-disrupting protocol on the bacterial load of equine wound samples is presently lacking.

The goal of this study was to correlate a clinical assessment, a quantitative swab, semi-quantitative swab and a swab for cytology with the bacterial load of an open wound as determined with a quantitative biopsy in horses. Additionally, the impact of a biofilm-disrupting protocol on the bacterial counts of equine wound samples with histologically observed biofilms was tested. The hypothesis was that both a quantitative and semi-quantitative swab would be a valid non-invasive alternative for a quantitative tissue biopsy to quantify bacterial load in second intention healing wounds in horses. Additionally, it is expected that bacterial counts will increase after a biofilm-disrupting protocol is performed on samples taken from wounds with a histologically observed biofilm.

Materials and methods

Following ethical committee approval on 8th May 2013 (approval number 2013-65), horses admitted to the Faculty of Veterinary Medicine (Ghent University) were screened for inclusion in this study (May 2013 to February 2015). To be included in the study, horses had to have one or multiple traumatic second intention healing wounds with a minimal surface area of 4 square cm. The wounds had to be at a stage where they presented granulation tissue. Horses with a systemic condition (e.g. Cushing) or on medication (e.g. corticoids) that could influence the immune system were excluded. Owners signed an informed consent before commencement of the study.

Wound samples

Relevant data of the horse (age, presence of fever > 38.5° C) and of the wound (location and wound type) were recorded. Wound evaluation and sampling were performed by the first author during a bandage or cast change. For the bandage change, horses were sedated with detomidine hydrochloride (Detogesic, Zoetis, 10µg/kg) and butorphanol tartrate IV (Turbogesic, Zoetis, 10µg/kg). For the cast change, horses were placed under general anaesthesia. They were premedicated with romifidine hydrochloride (Sedivet, Boehringer Ingelheim, 80µg/kg) and morphine hydrochloride (Morphine HCl, Sterop, 100µg/kg) IV, followed by induction with ketamine hycrochloride (Anesketin, Dechra, 2.2 mg/kg) and midazolam IV (Dormicum, Roche, 60 µg/kg). Isoflurane (Isoflo, Abbot) in oxygen was used for anaesthetic maintenance.

The wound was evaluated for clinical signs of infection using a check list (table 1). The wound pH was measured by means of a pH stick (Panpeha n°112®, Novolab), which was

placed on the wound surface until completely soaked with wound fluid. The pH was determined using the dedicated legend.

Table 1. The checklist used to evaluate the wounds for signs of infection.

If the sign listed was present in the wound, the box next to the description was checked off. The rows with three check boxes distinguish between the different signs listed, while for the rows with one checkbox, it was checked if either one of the described signs was present.

Exudate (oozed through the third layer of the bandage) clear / sanguineous / purulent			
Red granulation tissue (beefy aspect)			
Yellow necrosis/slough/discoloration of the granulation tissue			
Black necrosis/slough/discoloration of the granulation tissue			
Exuberant granulation tissue			
Oedematous granulation tissue (glassy, shiny aspect)			
Friable granulation tissue (easily bleeding when probing the surface and base of the wound)			
Bone visible or felt with a probe			
Oedema around the wound/ part of the limb /entire limb			
Unpleasant odour			
Pain (it is difficult to touch the wound even when the horse is sedated)			

After the clinical assessment, a total of 3 swabs were taken using the Levine Method (Levine et al., 1976). The wound was first rinsed with a sterile saline solution (0.9% NaCl, in a 35 mL syringe with a 19 G needle) to eliminate surface contamination. Excess fluid was removed by gently blotting the wound surface with a dry sterile gauze. According to the Levine technique a sterile rayon tipped swab was moistened with a few drops of sterile saline solution (0.9% NaCl) and a sterile template delineating 1 cm^2 was placed on the approximate centre of the wound. Next, a swab was taken over this 1cm^2 during 5 seconds while rotating the swab between the thumb and the index finger over 360° with sufficient pressure to express tissue fluid. The swab was put into a sterile container without transport medium and labelled with a reference number and the date. The other 2 swabs were taken according to the same procedure, but the template delineating 1 cm^2 was slightly shifted to avoid that the same area was swabbed twice. The swabs were processed within 1 hour or placed into a refrigerator (4°C) for a maximum of 6 hours.

As a final step, a wound biopsy was taken. A 6 mm diameter punch biopsy was placed near the centre of the wound over viable tissue, while avoiding the areas that were swabbed earlier. The bottom of the biopsy was marked with sterile Indian ink and placed into a sterile container, which was labelled with a reference number and the date. The biopsies were processed within 1 hour or placed into a refrigerator (4°C) for a maximum of 6 hours.

Processing of the swab samples

One swab was processed using quantitative and semi-quantitative bacteriological procedures. Another swab underwent a vortex and sonication protocol before it was processed for quantitative and semi-quantitative procedures to assess the influence of a biofilm-disrupting protocol. The final swab was processed for cytology. The used techniques are described in detail below.

The rayon tip of the swab used for the quantitative bacteriological data was cut off with sterile scissors and put into a 1.5 mL Eppendorf tube filled with 1mL of sterile phosphate buffered saline (PBS). The tip was vortexed for 30 seconds and then serially diluted 10-fold in PBS. Next, 20 µL aliquots of each dilution were spot-plated onto a Columbia agar supplemented with 5 % sheep blood (Oxoid). The Columbia agar plates were incubated in a 5 % CO_2 enriched atmosphere at 37°C for 24 and 48 hours, after which the colony-forming units (CFU) were counted. The number of CFU per swab was then calculated taking the dilution into account.

The same swab tip was used to provide semi-quantitative bacteriological data. Twenty µL of the undiluted solution holding the swab was spotted and streaked out on the first quadrant of a Columbia agar plate with 5% sheep blood (Oxoid). Next, the other 3 quadrants were streaked out, each time using a sterile inoculation loop and crossing the inoculation lines of the former quadrant twice, thus serially diluting the original inoculation spot. The plate was incubated as described above, after which the bacterial growth in the quadrants was assessed. The bacterial burden was classified as scant (+), light (++), moderate (+++) or heavy (++++) depending on the presence of growth in the first, second, third or fourth quadrant respectively.

The rayon tip of the swab used to assess the influence of a biofilm disrupting protocol was also cut off with sterile scissors and put into a 1.5 mL Eppendorf tube filled with 1mL of sterile PBS. Next, the tip was vortexed for 30 seconds and then sonicated 30 seconds in a sonication bath (B5210, 47 kHz, Branson). This was repeated 2 times to release bacteria from a potential biofilm (Brackman et al., 2013). Next, the swab was processed for quantitative and semi-quantitative bacteriology as described above.

The third swab was processed for cytology. The swab was placed centrally on a glass slide and rolled back and forward, so the middle third of the glass was covered with a thin layer of wound exudate. Subsequently, the slide was air dried and Gram-stained. The cytology samples were examined under a light microscope (CX31, Olympus) at magnification 1000× with immersion oil. In ten randomly chosen 1000× high power fields (HPFs), the number of white blood cells was counted. Afterwards the mean number white blood cells per HPF were calculated.

Processing of the tissue samples

The tissue biopsy was cut aseptically into three pieces. One part was fixed in 4% formaldehyde for histology. The other two pieces were weighed aseptically, put into a 1.5 mL microcentrifuge tube filled with 1 mL of sterile PBS, and homogenized with a disposable tissue grinder (disposable pellet mixer 1.5 mL, VWR). One piece was vortexed for 30 seconds, and the other one underwent the vortex and sonication protocol as described for the swab samples. Both tissue suspensions were serially diluted 10-fold in PBS and each dilution was spot-plated on a Columbia agar with 5 % sheep blood (Oxoid). The plates were incubated as described above, after which the CFU were counted. This number was converted into CFU per gram of tissue, by taking the dilution and the weight of the biopsy into account.

Histology

The formalin-fixed tissue sample was embedded in paraffin, sectioned in 4µm slices, and stained with four histological stains: hematoxylin and eosin stain (HE), Gram stain, Giemsa stain, and periodic acid shiff (PAS) stain. The stained samples were examined by light microscopy (CX 31, Olympus) at magnification 1000× for evidence of biofilms. Samples were considered positive for biofilms when both bacterial clusters and extracellular polymeric substance (EPS) (PAS +) were present (Stark et al., 1999; James et al., 2008).

Statistical analysis

Statistical analysis was performed with SPSS statistics 20 (IBM). The quantitative tissue biopsy that was not vortexed and sonicated was used as reference standard to determine the true infection status of the wound. The wound was defined as infected if the biopsy had a value of >1 × 10^5 CFU per gram of tissue (Robson, 1997). A base 10 logarithmic transformation was performed on the quantitative data of the swabs and biopsies to achieve normality. To explore the relationship between quantitative swabs and quantitative biopsies a linear regression analysis was performed. The correlation between the semi-quantitative swabs and the quantitative biopsies was measured using a Spearman’s rank correlation. To

assess if clinical parameters (table 1) and the cytology swab (amount of white blood cells present) were associated with the log CFU found in the wounds, univariable linear models were performed first. Clinical parameters with a P-value of ≤ 0.2 were then included in a multivariable linear model. This model was further refined by stepwise elimination of predictive parameters with a P-value of > 0.05, until a final model for evaluation was reached. To investigate if the samples with a histologically confirmed biofilm had higher bacterial count if they were subjected to the vortex and sonication protocol, a general linear model was used with bacterial load as dependent variable and procedure (with or without sonication) as independent variable. A P-value of < 0.05 was considered to be statistically significant. All model assumptions were checked and met.

Results

Forty-seven horses were included and 50 second intention healing wounds were sampled. The mean age of the horses was 7 years (range 7 days – 25 years) and the median duration of the wounds was 19 days (range 3 days- 370 days). The most common wound causes were wire cuts (n = 10), getting caught in a fence (n = 4) or getting stuck in a stable during rolling (n = 4). Other miscellaneous causes were cuts by metal plates (n = 2), a kick from another horse (n = 1), a car accident (n = 1), pressure ulcers (n = 1), etc. Of 16 wounds the cause was unknown. Forty wounds were located at the distal limbs (at the level of or below hock and carpal joint), nine were located at the proximal limb and one wound was located at the thorax. Of the limb wounds (n = 49) 25 were located at the right hind limb, ten at the left hind limb; nine at the left front limb and five at the right front limb. Most horses were sampled once, but three horses were sampled twice because of a strong suspicion of a wound infection during their hospitalisation period (no progress in wound healing, wounds with friable granulation tissue and excessive exudate).

Only ten out of 50 wounds had a bacterial count of > 10^5 CFU/g tissue and in only five wounds histological evidence of a biofilm was found, of which four wounds showed bacterial counts of > 10^5 CFU/g tissue. The vortex and sonication protocol did not significantly increase bacterial counts of the swabs and biopsies in samples with a histologically confirmed biofilm. In table 2 an overview is given of the average CFU values for the quantitative biopsy, the quantitative swab and the semi-quantitative swab for the infected (n = 10) and non-infected wounds (n = 40). In table 3 an overview is given of the prevalence of the different parameters of the clinical assessment in infected and non-infected wounds.

Table 2. An overview of average colony forming units (CFU) values for the quantitative biopsy, the quantitative swab and the semi-quantitative swab

	Mean Log CFU [a] for the quantitative biopsy (±SD [b])	Mean Log CFU for the quantitative swab (±SD)	Mode for the semi-quantitative swab
Infected wounds (n = 10)	6.64±1.12	4.97±1.02	++
Non-infected wounds (n = 40)	3.18±0.91	2.82±1.27	+

[a] CFU, colony-forming units
[b] SD, standard deviation

Table 3. An overview of the prevalence of the different parameters of the clinical assessment in the infected and non-infected wounds

	Infected wounds (n= 10)	Non-infected wounds (n = 40)
clear exudate	1 (10%)	10 (25%)
sanguineous exudate	3 (30%)	11 (28%)
purulent exudate	3 (30%)	9 (23%)
Red granulation tissue	8 (80%)	24 (60%)
Yellow granulation tissue	8 (80%)	30 (75%)
Black granulation tissue	6 (60%)	10 (25%)
Exuberant granulation tissue	4 (40%)	16 (40%)
Oedematous granulation tissue	4 (40%)	14 (35%)
Friable granulation tissue	1 (10%)	14 (35%)
Bone visible or felt with a probe	4 (40%)	9 (23%)
Oedema around the wound	4 (40%)	21 (53%)
Oedema of part of the limb	5 (50%)	16 (40%)
Oedema of entire limb	0	2 (5%)
Unpleasant odour	1 (10%)	9 (23%)
Pain	1 (10%)	3 (8%)
Fever	1 (10%)	0

A high correlation ($P < 0.001$ $r = 0.747$) was shown between the outcome of the quantitative swabs (CFU per swab) and the biopsies (CFU per gram of tissue). The quantitative swabs and biopsies were linearly related ($P < 0.001$) by the regression function $Y = 1.121 + 0.846 X$ ($r^2 = 0.56$), where Y represents the log CFU of the quantitative biopsy and the X represents

the log CFU of the quantitative swabs (Fig. 1). This means that a value of approximately > 1.2 x 10^5 CFU per swab corresponds with the critical value of > 1 x 10^5 CFU per gram of tissue for the quantitative biopsy.

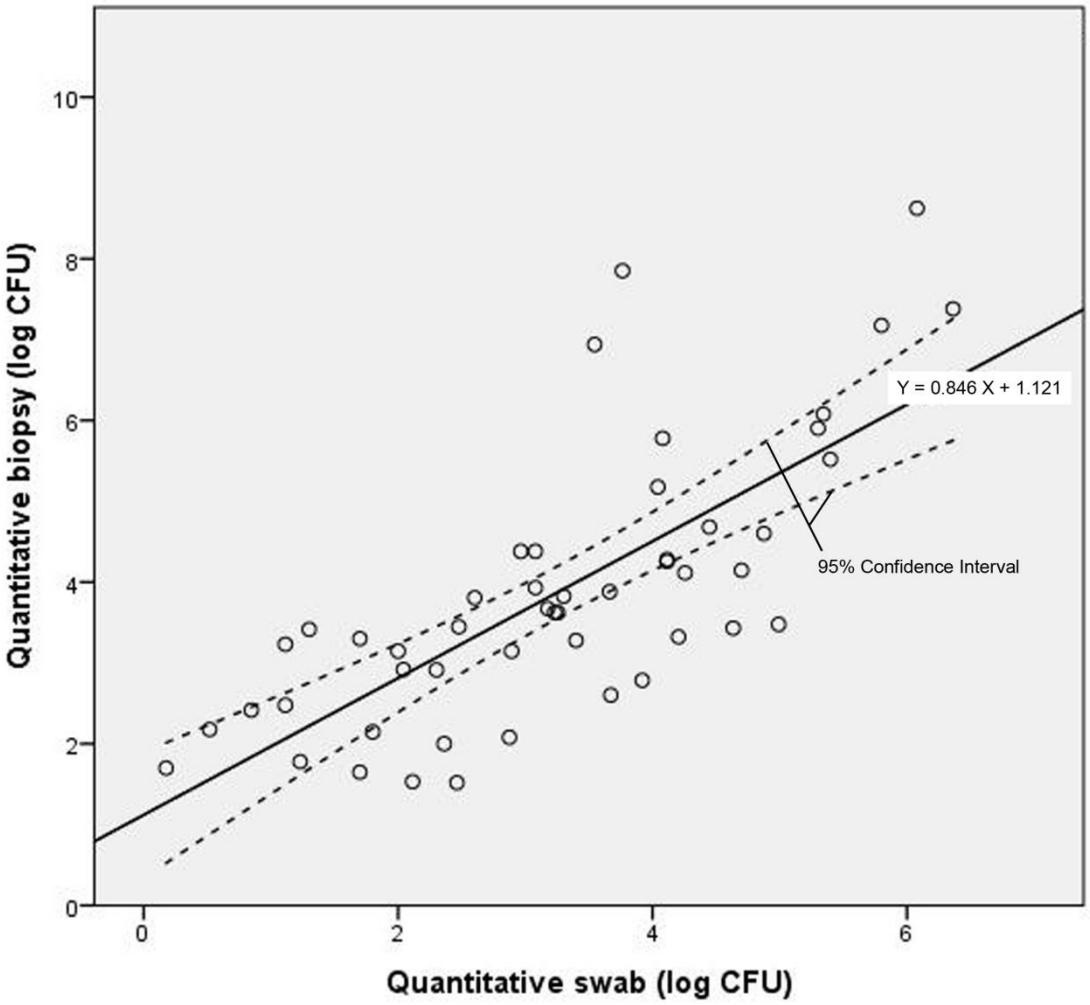

Figure 1. The correlation between the quantitative swabs and the quantitative biopsies.
The log CFU of the quantitative swab (X) is linearly related to the outcome of quantitative biopsy (Y) by the regression function Y= 1.121+0.846 X. The dotted lines indicate the 95% confidence intervals for the mean values of the quantitative swabs and biopsies.

The semi-quantitative swabs showed a significant but only moderate correlation to the quantitative biopsies ($P < 0.001$ $\rho = 0.524$).

Univariable linear models were performed as a first screening to evaluate if the swab for cytology or any of the parameters of the clinical assessment were significantly associated with higher CFU per gram of tissue or not. The swab for cytology, clear exudate, yellow granulation tissue, black granulation tissue, bone visible/palpable, oedema around the wound and oedema of part of the limb were included in the final multivariable linear model. Finally,

higher white blood cell counts for the swab for cytology were significantly associated with lower log CFU in the wounds (P = 0.02), whereas wounds with black granulation tissue showed significantly higher log CFU (P = 0.003).

Discussion

In this study, we investigated if a clinical assessment, a quantitative swab, a semi-quantitative swab and a swab for cytology of a second intention healing wound were correlated with the bacterial load as determined with a quantitative biopsy. Moreover, we tested the impact of a biofilm-disrupting protocol on the bacterial load of equine wound samples with histologically observed biofilms. It was expected that a quantitative and semi-quantitative swab could serve as an alternative for a quantitative tissue biopsy to quantify bacterial load in second intention healing wounds in horses. Moreover, we expected that the biofilm-disrupting protocol would increase the bacterial counts in samples with a histologically observed biofilm. Based on the results of this study, the quantitative swab seems a good alternative for a quantitative tissue biopsy to assess bacterial load in the wound. As mentioned in the results, a value of > 1.2 x 10^5 CFU per swab corresponded with the critical value of > 1 x 10^5 CFU per gram of tissue for the quantitative biopsy. These results are quite similar to the results of the study of Levine et al. (1976) in human wounds. They found that the critical value of > 1 x 10^5 CFU per gram of tissue of the biopsy corresponded with approximately > 1.5 x 10^5 CFU per swab. Therefore, it seems that a quantitative swab using the Levine technique can be used as an alternative for a quantitative biopsy when taking into account a slightly higher critical value to indicate infection. Moreover, the quantitative swab has the obvious advantage to be much less invasive and easier to process compared to the tissue biopsy.

The semi-quantitative swab in this study was only moderately correlated to the quantitative biopsy (ρ = 0.524), which made it unsuitable as an alternative for the quantitative biopsy. In human medicine, Ratliff and Rodeheaver (2002) reported that a semi- quantitative swab was correlated with a coefficient of r = 0.84 to a quantitative swab, and suggested that a semi-quantitative swab could be useful in the detection of wound infection. However, the semi-quantitative swab was not directly correlated to the quantitative biopsy, making it difficult to assess the value of semi-quantitative swab compared to the golden standard. More recently, Gardner et al. (2007) investigated the diagnostic value of the semi-quantitative swab by comparing it directly to the quantitative biopsy. They found that the semi-quantitative swab was a non-informative test and that a quantitative swab provides more meaningful information. The results of the present study confirm the findings of Gardner et al. (2007) albeit in equine wounds.

Wounds with black granulation tissue were significantly associated with higher log CFU per gram of tissue. Additionally, higher white blood cell counts for the swab for cytology were significantly associated with lower log CFU per gram of tissue. However, the number of samples with high white blood cell counts were low and the effect was small (for every increase in 1 log CFU per gram of tissue, there was decrease of 0.04 white blood cells), thus the value of the correlation between the swab for cytology and the quantitative biopsy is rather limited. In human medical literature, there is much discussion on the usefulness of clinical symptoms to identify wound infection (Gardner et al., 2001; Bowler, 2003; Serena et al., 2006; Woo and Sibbald, 2009). Gardner et al. (2001) stated that the classic signs (e.g. pain, erythema, oedema, heat and purulence) can be insufficient to detect infection in certain types of wounds, such as chronic wounds or wounds with high inflammatory response. Therefore, they investigated the validity of the classic signs and signs specific for secondary wounds (wounds left to heal by second intention) for the detection of chronic wound infection. They concluded that the signs specific for secondary wounds (i.e. serous exudate, delayed healing, discoloration of granulation tissue, friable granulation tissue, pocketing of the wound, foul odour and wound breakdown) were better to diagnose infection compared to the classic signs. However, Bowler (2003) reported that the diagnosis of wound infection should be based on primarily clinical signs and qualitative bacteriology and that the quantitative tissue biopsy only has a limited value. In contrast, Serena et al. (2006) found that 26% of the infected leg ulcers were missed using a pure clinical examination compared to a quantitative tissue biopsy. They also stated that high levels of bacteria have been proven to inhibit wound healing despite the absence of clinical signs. More recently, Woo and Sibbald (2009) validated a series of signs and symptoms based on the mnemonics (NERDS and STONEES) using semi-quantitative swabs. They concluded that when any three random signs were combined, a high sensitivity and specificity were obtained to detect moderate and heavy bacterial growth. However, the big limitation of the study of Woo and Sibbald (2009) was the use of semi-quantitative swabs to validate the signs and symptoms instead of the golden standard, i.e. a quantitative tissue biopsy, making the results of their study difficult to interpret.

The use of clinical signs to diagnose wound infection in horses is also a disputable matter. Horses have an aberrant wound healing at the distal limbs, comparable to chronic wounds in humans such as venous and arterial leg ulcers and wounds of diabetics (Theoret, 2008). The inflammatory response at the equine distal limbs has a slow onset, does not show a high peak and keeps persisting with a chronic inflammation as result (Wilmink, 2008). Consequently, this chronic inflammation leads to clinical signs usually associated with infection (discoloured or oedematous granulation tissue, exuberant or friable granulation tissue, high amount of exudate, etc...), even though a high bacterial burden is not always

present. Moreover, the skin of horses is often pigmented and hairy and wounds are often left to heal by second intention, which makes the classic signs of infection less suitable for evaluating wounds in horses. Therefore, to the authors' opinion, a quantitative tissue biopsy or swab has a high value to help diagnose wound infection in an objective way in horses, at least until the new developments in objectively diagnosing wound infection (e.g. enzyme detection, rt-PCR, etc...) (Tegl et al., 2015) become more suitable and available for routine use. Moreover, the 10^5 guideline is especially useful for research because it is a more objective technique than purely a clinical examination. Therefore, both in equine practice and research, a clinical assessment should be complemented with quantitative bacteriology.

It is generally recognized that there is an interaction between the bacteria present in a wound and the host. Depending on the amount of bacteria present in the wound, their virulence and the host's immune response, the wound is considered to be contaminated, colonized, critically colonized of infected (Edwards and Harding, 2004). Thus, a threshold value to define infection can come across as rather arbitrary. However, despite the 10^5 CFU/g tissue rule being increasingly questioned (Bowler, 2003; Kallstrom, 2014), it is still clinically relevant (Robson, 2003). Extensive research of Robson (1997; 2003) on a wide variety of wounds (chronic wounds after skin grafting and delayed closure, burn wounds, venous ulcers…) has demonstrated impeded wound healing when the bacterial load exceeded this threshold. Moreover, as mentioned by Robson (2003): 'The numerical distribution, like all biologic phenomena, forms a bell-shaped curve. Therefore, some infections might be expected to occur with fewer numbers of bacteria, and occasionally, no infection will be present when large numbers of bacteria are noted'. However, the authors are convinced that, to date, the 10^5 rule is the best objective measure to quantify bacterial load in a wound and is far less variable than the use of purely clinical signs. This especially applies to horses, with their disturbed inflammatory response in the distal limbs.

In this study, only 20% of the wounds were infected. Interestingly, the non-infected wounds displayed relatively more, albeit not significantly, friable granulation tissue and unpleasant odour. This can potentially be explained by the use of certain dressings during the wound treatment such as calcium alginate dressings, which give a wound an unpleasant odour independent of its bacterial load. These dressings also actively stimulate granulation tissue formation and the inflammatory response, which could also explain the higher presence of friable granulation tissue (Wilmink, 2008). Unfortunately, the primary dressing used before wound sampling was not recorded during this study.

The clinical assessment and the evaluation of the equine wound samples were all performed by the first author. However, because the evaluation was always done by the same person,

no variation was present in the interpretation of the assessments between the different wounds. Therefore, the comparison between the different wounds is valid. However, to increase objectivity of especially the clinical assessment, the evaluations would have been better performed by two or more independent investigators.

In this study, histological evidence for a biofilm was present in four out of ten infected wounds (40%) and in one non-infected wound. This is lower than the results of Westgate et al. (2011). Possible explanations for the difference between the study of Westgate et al. (2011) and our study can be found in the type of wounds sampled (chronic wounds in the study of Westgate et al. (2011) vs. all types of second intention healing wounds in our study), the location of the wound sampling (edge of the wound vs. centre of the wound) and biopsy technique (8 mm vs. one third of a 6mm biopsy). However, similar to the study of Westgate et al. (2011), this study confirms the presence of biofilms in equine second intention healing wounds, which can explain the delayed wound healing in some wounds. The vortex and sonication protocol did not seem to influence quantitative bacteriology in equine wound samples with a histologically confirmed biofilm. However, the number of samples with a biofilm was low, which makes it difficult to interpret the results. Moreover, the authors acknowledge that the use of light microscopy is not the most sensitive technique to visualize biofilms, but it has been successfully used in other studies (Harrison-Balestra et al., 2003; Westgate et al., 2011). In this study, the biofilms were imbedded in the tissue just below the wound surface (Fig. 2), which could explain why the bacterial burden of the swabs were not influenced by the biofilm-disrupting protocol because the swabs sample the wound surface. An explanation for the lack of influence of the biofilm-disrupting protocol on the bacterial load of the tissue biopsies could be that the homogenization of the tissue is apparently sufficient to release all the bacteria from the biofilms. However, it is also possible that this protocol is not the best technique to release bacteria from biofilms in wounds, despite it has been successfully used in an in vitro chronic wound model (Brackman et al., 2013).

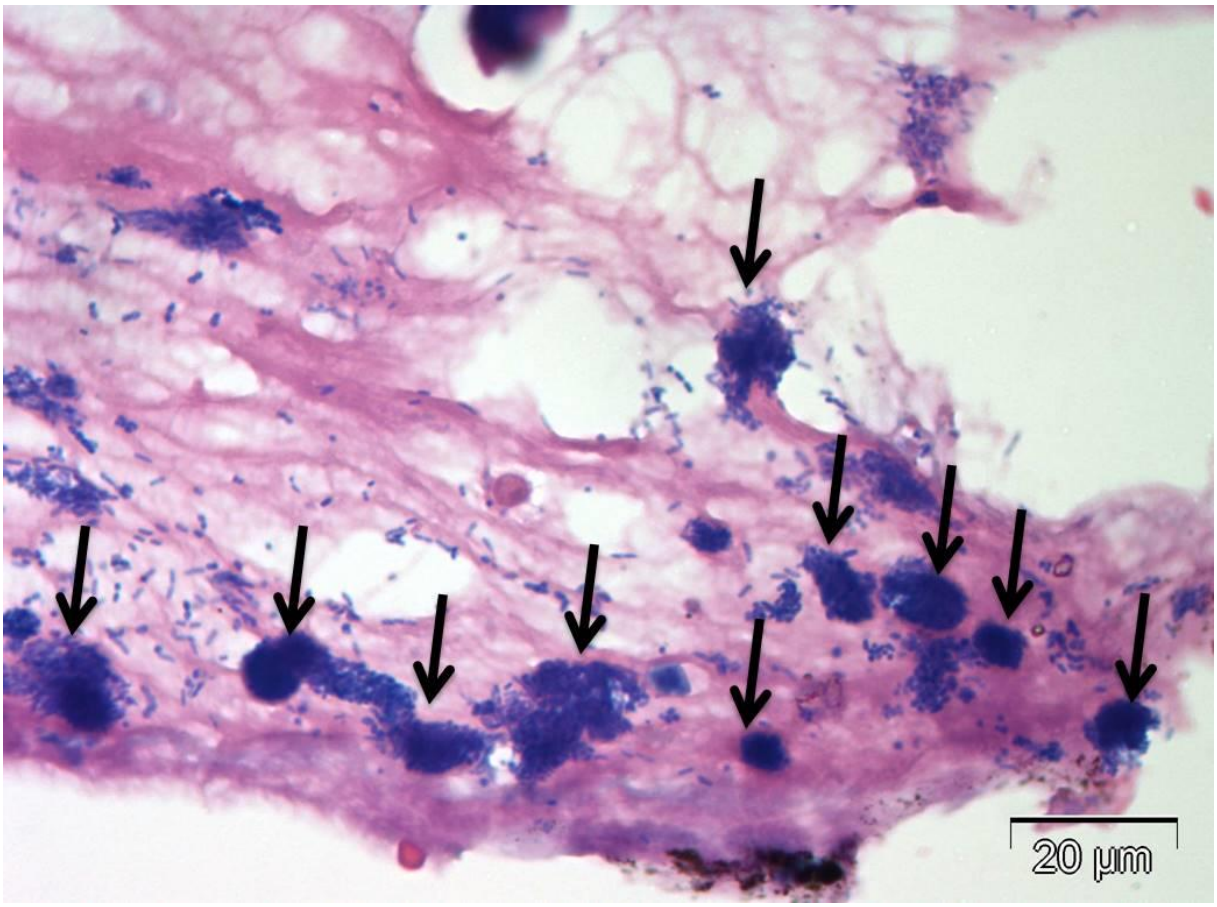

Figure 2: A Giemsa stain of a wound sample with histological evidence of a biofilm (1000x).
The wound surface is oriented towards the bottom of the picture. The bacteria (blue coloured) are present in a thumbprint fashion just below the wound surface indicating the presence of a biofilm (black arrows).

Conclusion

Based on the results of this study, a quantitative swab taken according to the Levine technique seems a good non-invasive alternative to a quantitative tissue biopsy to quantify bacterial load in second intention healing wounds in horses.

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THE ANTIBACTERIAL EFFECT OF NEGATIVE PRESSURE WOUND THERAPY USING THREE DIFFERENT FOAMS IN AN EQUINE PERFUSED EX VIVO WOUND MODEL

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Summary

The objective of this study was to compare the antibacterial effect of negative pressure wound therapy (NPWT) using three different foams to a standard antibacterial dressing in an equine perfused *ex vivo* wound model. An abdominal musculocutaneous flap was collected from six equine cadavers. Four circular wounds of 5 cm diameter were created per flap and were inoculated with methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa.* After an incubation period, the wounds were assigned to one of following four treatment groups: (1) NPWT using a silver impregnated polyurethane foam (NPWT-AgPU), (2) NPWT using a normal polyurethane foam (NPWT-PU), (3) NPWT using a polyvinyl alcohol foam (NPWT-PVA) or (4) reference treatment (R) using a non-adherent antimicrobial dressing with polyhexamethylene biguanide without NPWT. An 8 mm punch biopsy was obtained from each wound before application of the treatments (T0) and then every 6 hours during the 24 hour treatment protocol (T6, T12, T18, T24) to calculate the bacterial load.

For *P. aeruginosa*, NPWT-PVA resulted in a significantly lower bacterial load from T6 to T24 compared to the other three treatments. The bacterial load with NPWT-AgPU was lower than with C and NPWT-PU from T6 to T24 but only significantly so at T12. For MRSA, the bacterial load with NPWT-PVA was significantly lower compared to the other treatments from time point T6 on. NPWT-PVA gave the greatest reduction in bacterial load, but should be tested *in vivo* to assess its influences on wound healing.

Introduction

Negative pressure wound therapy (NPWT) is an established treatment to enhance wound healing in human medicine, and is gaining popularity in veterinary medicine (Demaria et al., 2011; Krug et al., 2011; Mouës et al., 2011; Vig et al., 2011; Birke-Sorenson et al., 2011). In veterinary medicine however, research on NPWT and its feasibility for clinical use is just starting. Especially in equine medicine only a limited number of publications are available and they mainly entail case reports (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Jordana et al., 2011). Nevertheless, NPWT could be very valuable to equine medicine because its mechanisms of action would address common problems associated with equine wound healing including massive tissue loss, poor perfusion and a weak but protracted inflammatory response.

According to Mouës et al. (2011), NPWT results in an increase in blood flow, promotion of angiogenesis, induction of cell proliferation, reduction of wound area in certain types of wounds and a modulation of inhibitory contents in wound fluid such as matrix metalloproteinases. Nevertheless, the effect of NPWT on contamination or infection of wounds with different bacterial species is still controversial both in human and veterinary medicine (Morykwas et al., 1997; Mouës et al., 2004; Weed et al., 2004; Braakenburg et al., 2006; Mouës et al., 2011; Yusuf et al., 2013; Patmo et al., 2014). In the original study of Morykwas et al. (1997), NPWT induced a significantly faster drop of bacterial load compared to saline-gauze treated control/reference wounds. In contrast, Weed et al. (2004) found that during NPWT the bacterial load increased, while the study of Mouës et al. (2004) reported that the overall bacterial load was stable during NPWT, but that there was a decrease of nonfermentative gram negative rods and an increase of *Staphylococcus aureus*. The study of Braakenburg et al. (2006) supported the finding that NPWT did not enhance bacterial clearance. Based on these results, it is impossible to formulate an unequivocal conclusion on the effect of NPWT on bacterial load in wounds.

Additionally, there is a lack of studies that investigate the influence of the different types of dressings used with NPWT on the bacterial load in wounds. To the authors' knowledge, only Yusuf et al. (2013) reported a difference in bacterial load in vivo between black polyurethane and white polyvinyl alcohol foam, with a significantly lower load in the polyurethane compared to the polyvinyl alcohol foams. However, Yusuf et al. (2013) did not directly investigate the bacterial load in the wounds and hypothesized that the load in the foams was representative for the bacterial load in the wounds.

To gain more insight in the effect NPWT has on different bacterial species and wound bacterial load, a study was conducted using an equine perfused ex vivo wound model based

on the in vitro model of Assadian et al. (2010), which allowed monitoring bacterial load in wounds with sequential biopsies. The goal was to compare the antibacterial effect of NPWT using three different foams namely a silver impregnated polyurethane foam, a standard polyurethane foam and a polyvinyl alcohol foam to a standard antibacterial dressing without negative pressure. The hypotheses were that in this model NPWT with the different foam dressings would lower the bacterial load more compared to the standard antibacterial dressing without negative pressure and that NPWT using a silver impregnated foam, which is specially developed to treat heavily contaminated wounds, would give the greatest reduction in the bacterial load compared to NPWT using the other foams.

Materials and methods

Six horses euthanized for clinical reasons other than colic or septicaemia were used to collect one abdominal musculocutaneous flap each. Owners gave their consent that the cadavers could be used for scientific purposes. All procedures were approved by the institution's animal care and use program. Just after the lethal injection with a combination product containing embutramide, the horses received a dose of heparin (150 IU/kg IV) to avoid blood coagulation in the capillaries of the flap.

Perfused ex vivo wound model

After euthanasia, the cadavers were positioned in lateral recumbency. The hairs overlying the *rectus abdominis* muscle were first clipped and then shaved with razor blades. The abdominal area was thoroughly scrubbed with chlorhexidine digluconate soap for 10 minutes until the gauzes where free from visible contamination, and afterwards disinfected with 70% ethanol. Next, a 25 by 35 cm flap of the rectus abdominis muscle and overlying skin at the level of the superficial epigastric vein and artery, was aseptically removed and placed on a sterile plate. Care was taken not to involve the linea alba in the musculocutaneous flap and the flap was transported to the laboratory within 5 minutes after removal to avoid contamination.

In the laboratory, the flap was kept at room temperature. A 18G catheter was inserted into the superficial epigastric artery and secured with a Chinese finger trap suture. The catheter was connected to a sterile saline solution (0.9 % NaCl) infusion bag at room temperature. The leaking blood vessels at the periphery and bottom of the flap were closed with simple interrupted sutures (Fig. 1) and the rate of the infusion was set at 10 gtts/min to prevent the tissue from desiccating. Four circular wounds of 5 cm diameter were created on the flap by removing skin, cutaneous trunci muscle and the outer layer of the rectus sheath. Bacterial stock suspensions of two common equine wound pathogens, namely MRSA and

Pseudomonas aeruginosa, isolated from equine wound patients, were prepared. The bacteria were identified through morphology and biochemical testing and the antibiotic resistance of the MRSA was tested by means of an antibiogram. Pure cultures of each of the bacteria were scraped from an overnight culture on Columbia agar plates supplemented with 5% sheep blood using sterile cotton tipped swabs and suspended in sterile phosphate buffered saline (PBS) as suspension solution. The turbidity of each suspension was adjusted to 1 McFarland with a densitometer. Next, both bacterial suspensions were diluted 1:300 in PBS, so a final concentration of approximately 10^6 colony-forming units per mL was achieved. Each wound on the musculocutaneous flap was inoculated with 1 mL of the MRSA and 1 mL of the *P. aeruginosa* suspension by carefully applying the suspensions with an air displacement micropipette. The entire flap was incubated for 1 hour at 37°C and 5 % CO2. During incubation, the sterile saline infusion was temporarily disconnected. A total of six replicates were performed.

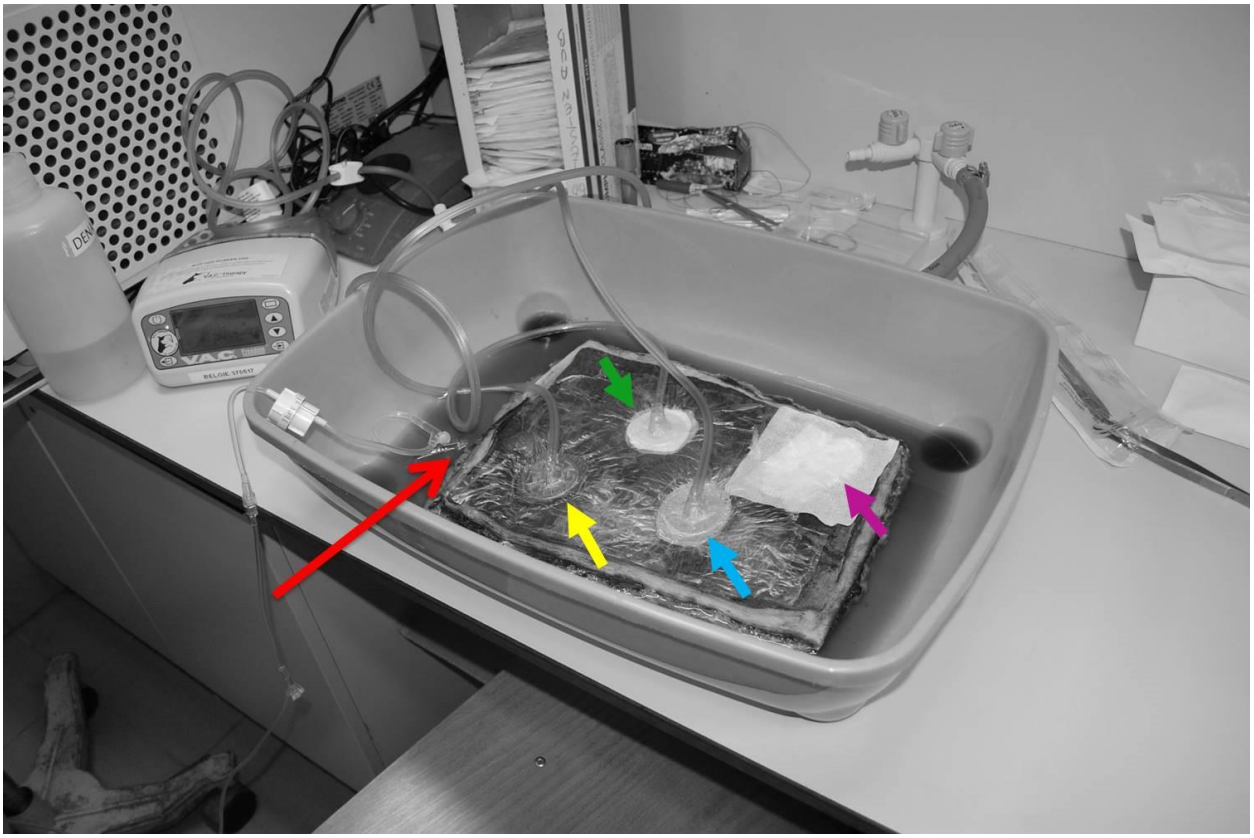

Figure 1: An overview of the experimental setup with the abdominal musculocutaneous flap, on which the 4 different treatments are applied.

From left to right (1) negative pressure wound therapy (NPWT) using a normal polyurethane foam (yellow arrow), (2) NPWT using a polyvinyl alcohol foam (green arrow), (3) NPWT using a silver impregnated polyurethane foam (blue arrow), and (4) the reference treatment using a non-adherent antimicrobial dressing with polyhexamethylene biguanide, without NPWT (purple arrow). The catheter inserted in the superficial epigastric artery is indicated with a red arrow.

Wound treatments

After an incubation period of 1 hour, the catheter was reconnected to the sterile saline infusion and the wounds of each flap were randomly assigned to one of the following four treatment groups: (1) NPWT using a silver impregnated polyurethane foam (V.A.C. GranuFoam silver™, KCI medical) (NPWT-AgPU) , (2) NPWT using a standard polyurethane foam (V.A.C. GranuFoam™, KCI medical) (NPWT-PU), (3) NPWT using a polyvinyl alcohol foam (V.A.C. WhiteFoam™, KCI medical) (NPWT-PVA) or (4) reference (R) treatment using a non-adherent antimicrobial dressing with polyhexamethylene biguanide (Telfa AMD™, Instrulife) without NPWT.

Before treatment application, the excess bacterial suspension was removed from the wounds with a dry sterile gauze and the skin around the wounds was disinfected with alcohol to prevent contamination. Next, the different dressings were cut into a circular shape (5 cm diameter) and placed in the designated wounds. The surrounding skin was then degreased with ether and adhesive spray was applied. The three wounds assigned to NPWT (NPWT-AgPU, NPWT-PU, NPWT-PVA) were covered with an occlusive polyurethane foil (V.A.C. drape, KCI medical). A 2 cm opening was cut in the occlusive foils over each of these three wounds and a suction pad (SensaT.R.A.C. pad, KCI medical) was applied. Next, the suction pads were connected to the canister of the NPWT system by means of Y-connectors. The negative pressure was set at 125 mmHg in continuous mode (Fig 1). The remaining C wound was covered with an adhesive fabric for dressing retention (Hypafix™, Instrulife, Oostkamp).

Microbiological analysis

In order to determine the bacterial load, an 8 mm punch biopsy was obtained from each wound before application of the treatments (T0) and then every 6 hours during the 24 hour treatment protocol (T6, T12, T18, T24). This required the dressings to be temporarily removed and repositioned afterwards. The biopsies were stored at 4°C and processed for standard quantitative bacteriological analysis in the laboratory within 12 hours of collection. Briefly, the biopsies were aseptically weighed and homogenized in 1 mL PBS using a disposable tissue grinder (pestle and microtube combo, VWR international BVBA). Serial dilutions were spot plated on Columbia Colistin and Nalidixic acid agar (CNA) and MacConkey agar and the plates were incubated for 24 hours at 37°C and 5% CO2. The CNA and MacConkey plates were used to calculate the colony forming units (CFU) per gram of tissue for MRSA and *P. aeruginosa*, respectively. The bacterial load of the wounds at T0 was considered the baseline value to which the other values during the treatment protocol were compared.

Statistical analysis

Statistical software (IBM SPSS statistics 20, IBM Corp.) was used for all statistical analyses. A base 10 logarithmic transformation was performed on the data to approach normality. The data was checked for normality by using QQ-plots. Sphericity was tested using Mauchly's test of sphericity and when sphericity could not be assumed, P-values were corrected using the Greenhouse-Geisser method. An analysis of variance for repeated measures was performed with bacteria, treatment, time and their interaction as fixed factors and log bacterial load as dependent variable. Significant interactions were further investigated by splitting the file according to one of the interacting factors and performing an analysis of variance for repeated measures with the other factor as explaining variable. All multiple comparisons were performed using Tukey's method. A P-value of ≤ 0.05 was considered to be statistically significant. If the bacterial load of a biopsy could not be calculated because it was lower than the limit of detection of the quantitative bacteriological method, the missing data was replaced by one-half the detection limit value as described in the study of Antweiler and Taylor (2008).

Results

A significant effect of bacterium was found at T0 ($P < 0.001$). Therefore, further analysis of the data was performed separately for the two bacterial species. Within a bacterial species no significant difference in bacterial load was found at T0 ($P = 0.58$) for the different treatments. Hence, the bacterial load of the other time points (T6, T12, T18, T24) could be compared to this baseline value without converting the data into percentages.

For *P. aeruginosa*, NPWT-PVA resulted in a significantly lower bacterial load at T6, T12, T18 and T24 compared to the other treatments (with $P < 0.001$ for R and NPWT-PU and $P < 0.05$ for NPWT-AgPU). The bacterial load in the NPWT-AgPU treated wounds was lower than the bacterial load of the R and NPWT-PU treated wounds from T6 to T24 but this was only significant at T12 ($P = 0.033$ for R and $P = 0.029$ for NPWT-PU) (Fig. 2). For MRSA, NPWT-PVA also resulted in a significantly lower bacterial load at every time point from T6 on, compared to the three other treatments (Fig. 3) (with $P \leq 0.001$)

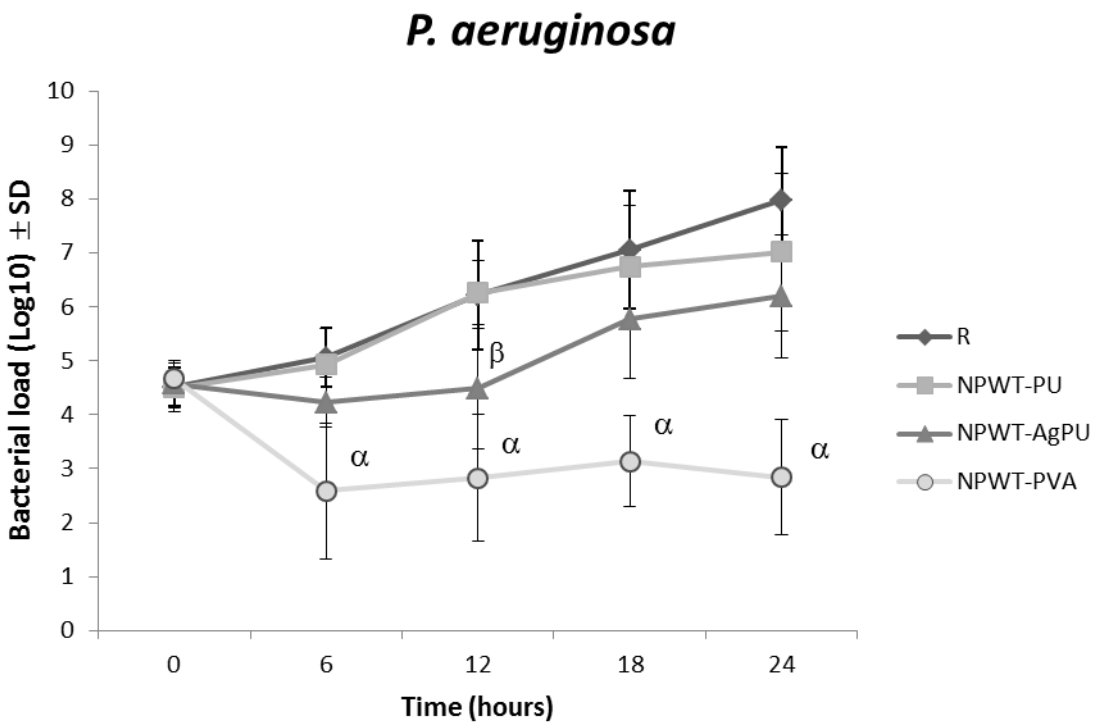

Figure 2. The evolution of the mean bacterial load in log10 of *P. aeruginosa* over time for the different treatments.

α,β: value differs significantly compared to the other treatments at the same time point with P <0.05. SD: standard deviation, R: reference treatment, NPWT-PU: negative pressure wound therapy using a normal polyurethane foam, NPWT-AgPU; negative pressure wound therapy using a silver impregnated polyurethane foam, NPWT-PVA: negative pressure wound therapy using a polyvinyl alcohol foam.

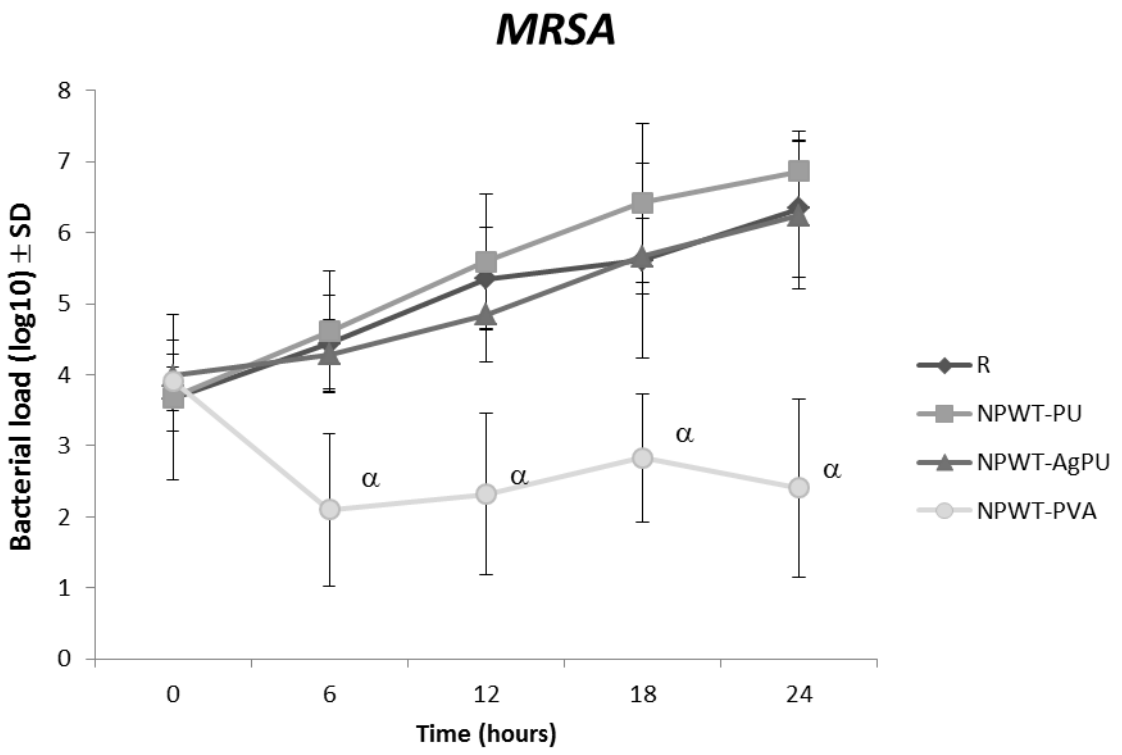

Figure 3. The evolution of the mean bacterial load in log10 of MRSA over time for the different treatments.

α: value differs significantly compared to the other treatments at the same time point with *P* <0.05. SD: standard deviation R: reference treatment, NPWT-PU: negative pressure wound therapy using a normal polyurethane foam, NPWT-AgPU; negative pressure wound therapy using a silver impregnated polyurethane foam, NPWT-PVA: negative pressure wound therapy using a polyvinyl alcohol foam

Comparing bacterial loads of *P. aeruginosa* between T0 and other time points within a treatment, no significant changes were seen for the NPWT-AgPU treated wounds. In the reference wounds (R), a significant increase in bacterial load was seen at time points T18 and T24 compared to T0 (P = 0.007 an P = 0.001 respectively). In the NPWT-PU treated wounds, the load was significantly higher at time points T12 (P = 0.002) and T18 (P = 0.008) compared to T0. In contrast, the bacterial load in the NPWT-PVA treated wounds was significantly lower at time point T18 (P = 0.026) compared to T0.

In the MRSA infected wounds, a significant increase in bacterial load was seen for the NPWT-AgPU treated wounds at time points T18 (P = 0.002) and T24 (P = 0.003) compared to T0. In the reference wounds (R), the bacterial load was significantly higher at time points

T12 (P = 0.039) and T24 (P = 0.019) compared to T0. In the NPWT-PU treated wounds, the bacterial load was significantly higher at time points T12 to T24 (P = 0.001 for all three) compared to T0. No significant effect of time was seen for the NPWT-PVA treated wounds.

Discussion

Of all the evaluated treatments in this study, NPWT-PVA had the best antibacterial effect against both *P. aeruginosa* and MRSA. Additionally, the NPWT-PVA treatment resulted in a decrease of bacterial load over time for *P. aeruginosa* while the MRSA load remained stable. The NPWT-AgPU treatment had a reasonable antibacterial effect against *P. aeruginosa* compared to NPWT-PU and R, and gave a stable load over time for this species. However, for MRSA infected wounds, the NPWT-AgPU treatment did not differ in antibacterial capacity from the NPWT-PU and the reference treatment. Moreover, the MRSA load increased over time with NPWT-AgPU. NPWT-PU and the R dressing had an unexpectedly limited effect in slowing down bacterial growth and the bacterial load of both bacterial species increased over time with these treatments.

The NPWT-PVA treatment had a greater antibacterial effect compared to the NPWT-AgPU treatment, which is interesting because the silver impregnated polyurethane foam has been specifically developed for treating heavily contaminated wounds with NPWT (Payne and Ambrosio, 2009). Possibly, the continuous negative pressure exerted on the wound cleared out the silver ions before they have time to destroy to bacteria. Additionally, the MRSA strain used in this study might have been silver resistant, which is an increasing problem in human and veterinary medicine in general (Silver, 2003; Woods et al., 2009).

According to the manufacturer, the greater antibacterial effect of the polyvinyl alcohol foam could be attributed to the presence of very low concentrations of formaldehyde in this foam. The formaldehyde is not an intentional component of the foam, but is formed during the gamma radiation sterilization process. Formaldehyde interacts with primary amines to form Schiff bases, with amides to form hydroxymethyl compounds (protein denaturation) (Thavarajah, et al., 2012) and has a low pH (2.8-4.0), which enhances the detrimental effect against bacteria. However, formaldehyde is not only toxic for bacteria, but also for cells in general. The presence of formaldehyde in the polyvinyl alcohol foams could therefore possibly negatively influence the formation of granulation tissue and epithelisation in wounds. To the authors knowledge, no studies in human or veterinary medicine have been performed which compare the rate of granulation tissue formation between polyurethane and polyvinyl alcohol foams or which evaluate the effect of polyvinyl alcohol foams on wound healing in a controlled manner. The manufacturer also mentioned that a decrease of *Pseudomonas* in suspension was seen with a concurring increase of *Pseudomonas* in polyvinyl alcohol foams,

suggesting a certain cyto-adhesive effect of the foam (unpublished data). This corresponds with the findings of Yusuf et al. (2013), who found that polyvinyl alcohol foams contained significantly more bacteria than normal polyurethane foams after applying them to chronic wounds in humans.

The fact that NPWT-PU and the R dressing had only a limited effect in slowing down the bacterial growth strengthens the hypothesis that the potential anti-bacterial effect of NPWT is not due to suctioning of bacteria out of the wound, but to the type of primary dressing or possibly the immune-modulating effect and increase in blood flow of the wound. Almost all previous studies which investigated the effect of NPWT on bacterial clearance of wounds used normal polyurethane foams as primary dressing, with varying results (Morykwas et al., 1997; Mouës et al., 2004; Braakenburg et al., 2006; Assadian et al., 2010; Steingrimsson et al., 2012). Based on the results of the present study, the use of this foam would not be the primary choice to treat heavily contaminated wounds, while the polyvinyl alcohol foam would be. Therefore, it would be interesting to investigate the effect of a polyvinyl alcohol foam in a clinical setting.

Due to ethical considerations, the authors decided to use an *ex vivo* wound model in the present study instead of clinical or experimental wounds. While this may be considered a limitation of the study, the *ex vivo* wound model made it possible to evaluate the antibacterial effect of NPWT with different foams without interference of the immune system. The equine perfused *ex vivo* wound model used in this study was based on the one of Assadian et al. (2010), who used raw porcine meat without skin. The model used in the present study consisted of musculocutaneous flaps of recently euthanized horses, so tissue decay was minimized and the integrity of the subcutaneous connection and the patency of the capillaries was preserved. To make the model more representative for clinical wounds, the skin was preserved and the tissues were perfused to mimic a potential flushing effect of the wound fluid. One container was used to recuperate the fluid from all NPWT treated wounds, which made it impossible to differentiate the amount of fluid per wound. The mean amount of fluid recuperated from all three NPWT treated wounds was around 15 mL over the 24 hour evaluation period.

In the original study of Morykwas et al. (1997), the enhanced bacterial clearance of the wounds by NPWT was attributed to the increased blood flow, which ameliorates the resistance of compromised tissue to infection and improves oxygenation. Morykwas et al. (1997) stated that the higher local oxygen level diminishes growth of anaerobes and enhances the oxidative bursts of the neutrophils, which destroy bacteria. Additionally, Assadian et al. (2010) demonstrated that bacterial load in wounds is not influenced by the

mere suction effect of NPWT. They found no significant difference in bacterial load between the use of the polyurethane dressing with and without negative pressure, so they stated that the influence of NPWT on bacterial clearance seen in other studies is probably the result of an immune-modulating effect. The use of an *ex vivo* model in the present study eliminated the immune system and made it possible to evaluate the effect of purely NPWT with three different primary dressings on different bacterial species. Nevertheless, it does not allow to differentiate between the antibacterial effect of the dressings on the one hand and the suction effect of the NPWT system on the other hand. In order to evaluate this, extra wounds would have to be created, on which the NPWT dressings only (without negative pressure) would have to be applied as has been done by Assadian et al. (2010). This is however not clinically relevant, since NPWT dressings are always used together with negative pressure in clinical circumstances. Therefore, a non-adherent antimicrobial dressing with polyhexamethylene biguanide was chosen as a reference treatment, because this treatment is often used in the institution of the authors and was deemed more clinically relevant.

For practical reasons, only two bacterial species were used in this experiment. While it would be beneficial to investigate more species in the future, the bacterial species used here are common equine wound pathogens. Both a gram positive and a gram negative species were used to represent normal wound flora (Westgate et al., 2011). It is interesting to see that even with only two bacterial species a clear difference of antibacterial effect was shown between the different treatments. In the *in vivo* studies, variable results were reported on the antibacterial effect of NPWT (Morykwas et al., 1997; Mouës et al., 2004; Weed et al., 2004; Braakenburg et al., 2006). However, differences in conclusions between these studies could be explained by the different study designs, methods of sample collection and types of wounds. For example, the original study of Morykwas et al. (1997) was an animal study with experimentally induced wounds infected with 2 gram positive bacterial species and biopsies were used for quantitative bacteriology. In that study, a significantly faster drop of bacterial load was noted with NPWT (using a polyurethane foam dressing) compared to saline-gauze treated control/reference wounds. In contrast, Weed et al. (2004) found with their retrospective study that NPWT increased bacterial load. That study included a mix of chronic and acute wounds and used quantitative swabs for the follow up of the bacterial load. A control/reference group was not used, but bacterial load was compared before, during and/or after NPWT (type of dressing not mentioned). The study of Mouës et al. (2004) was a prospective randomized trial with a mix of acute and chronic wounds. Biopsies were used for quantitative bacteriology and the control/reference group consisted out of moist gauze treated wounds. They concluded that NPWT (using a polyurethane foam dressing) gave a stable overall bacterial load, but that there was a decrease of nonfermentative gram negative

rods and an increase of *Staphylococcus aureus*. Braakenburg et al. (2006) also used a randomized controlled trial and a mix of acute and chronic wounds, but for bacteriology a semi-quantitative procedure was used (absence or presence of bacteria). In that study NPWT (using a polyurethane foam dressing) did not enhance bacterial clearance compared to control/reference treatment. Our study was an *ex vivo* experiment using different NPWT dressings and a non-adherent antimicrobial dressing with polyhexamethylene biguanide as a reference dressing. Biopsies were used for follow up of the bacterial load. Our results showed that the black polyurethane foam dressing, which was the most common type used in the *in vivo* studies, did not decrease the bacterial load and even increased it over time, thus concurring with the results of Weed et al. (2004). However, comparing the results of an ex vivo wound model with *in vivo* studies is difficult. Our results give an indication of the antibacterial effect of the different dressings used with NPWT, but these results have to be confirmed in an *in vivo* study which can also assess the effect the different dressings have on wound healing. This is especially important for the silver impregnated polyurethane foam and the polyvinyl alcohol foam dressings, which have not been tested in randomized controlled trials yet.

In this study, we did not include a negative control to evaluate bacterial load over time without treatment for practical reasons. However, since both the chosen bacterial species grew easily on Columbia agar plates supplemented with 5% sheep blood, It was considered highly unlikely the bacteria would not grow in muscle and skin tissue. After all, these tissues provide a far more favorable environment with an abundance of nutrients for bacterial growth than a standard agar plate.

Conclusion

In the *ex vivo* model, the capacity of NPWT to enhance bacterial clearance depended on the primary dressing used. In contrast to our hypothesis, NPWT using a polyvinyl alcohol foam gave the greatest reduction in bacterial load. NPWT using a silver impregnated polyurethane foam gave a steady state for *P. aeruginosa* over time and an increase of MRSA. NPWT using a polyurethane foam and the reference dressing both gave an increase of bacterial load over time. However, NPWT using a polyvinyl alcohol foam or silver impregnated polyurethane foam should be tested in an *in vivo* controlled trial to objectively assess the influences of these therapies on wound healing.

Acknowledgements

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THE CLINICAL RELEVENACE OF NEGATIVE PRESSURE WOUND THERAPY FOR THE TREATMENT OF SECOND INTENTION HEALING WOUNDS IN HORSES

THE EFFECT OF NEGATIVE PRESSURE WOUND THERAPY ON SECOND INTENTION HEALING OF ACUTE EQUINE DISTAL LIMB WOUNDS: A RANDOMIZED CONTROLLED EXPERIMENTAL STUDY

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Summary

The goal of this study was to assess the added value of negative pressure wound therapy (NPWT) on second intention healing of acute equine distal limb wounds compared to Ca-alginate dressings. Two circular 3.5 cm diameter wounds were created on both metacarpi in 5 horses. In the first 9 days, one limb was treated with NPWT, while the contralateral limb was treated with Ca-alginate dressings (reference). From day 9 to 71 both limbs were kept under bandage with hydrophilic polyurethane foam dressings. Over the course of healing, wound dimensions were measured, swabs were taken for quantitative bacteriology and blood flow was indirectly measured using a thermographic camera. At 5 time points, an 8 mm punch biopsy was taken for growth factor analysis, histological scoring of inflammation and repair, as well as immunohistochemical demonstration of B-cells, T-cells, macrophages, endothelium and myofibroblasts.

The wound area of NPWT wounds was significantly smaller compared to the reference wounds during the 9-days treatment period ($P = 0.005$) and in the early period after treatment ($P = 0.003$), but not in the late period after treatment. The maximum depth of the NPWT treated wounds was significantly larger compared to reference wounds during the treatment period ($P = 0.007$) and the early period after treatment ($P = 0.029$), but not in the late period after treatment.

The reference wounds had a significantly higher ($P = 0.044$) histological score for acute inflammation compared to the NPWT-treated wounds. However, in the NPWT wounds, significantly ($P = 0.008$) more macrophages were present. There was no significant difference in histological repair scores, growth factor concentrations, nor in the presence of B-cells, T-cells, myofibroblasts and neovascularisation.

Based on the results, no real advantage could be detected in the use of NPWT over Ca-alginate dressings for the treatment of acute distal limb wounds in horses.

Introduction

Horses are flight animals which makes them prone to traumatic wounds. These wounds are often left open to heal by second intention because of massive tissue loss, heavy bacterial contamination, high skin tension, a long duration from the onset of the injury or a combination of these scenarios (Theoret, 2008a). During second intention healing, the inflammatory response of distal limb wounds has a slow onset, does not show an effective peak and persists over time, leading to chronic inflammation and impeded wound healing (Wilmink, 2008). The course of the inflammatory response can explain many of the complications seen during the healing of equine distal limb wounds, such as exuberant granulation tissue formation, lack of wound contraction and frequent wound infection. This aberrant wound healing at the distal limbs of horses has similarities to chronic non-healing wounds in humans such as venous leg ulcers, and diabetic ulcers (Theoret, 2008a; Wilmink, 2014).

Negative pressure wound therapy (NPWT) is a technique that applies a continuous or intermittent negative pressure to the wound bed to expedite wound healing (Morykwas et al., 1997). It is frequently used in human medicine for chronic wounds (Vig et al., 2011) and could be interesting for treating distal limb wounds in horses. Indeed, NPWT increases local blood supply of the wound, stimulates angiogenesis and granulation tissue formation, reduces wound surface area and removes excess exudate in men (Mouës et al., 2011). Nevertheless, studies on the use of NPWT in horses are scarce and mainly consist of case reports (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Jordana et al., 2011) and, to the authors' knowledge, research on the influence of NPWT on equine distal limb wounds is lacking.

The aim of this study was to assess the added value of NPWT for treating second intention healing acute, non-inoculated distal limbs wounds of horses compared to the use of Ca-alginate dressings. The hypothesis was that NPWT would result in a faster decrease in wound dimensions, would increase the inflammatory response, improve local blood flow and decrease bacterial load compared to the Ca-alginate dressings.

Materials and Methods

Horses

Five warmblood horses (7-15 years of age) with both metacarpi free of scars were used in this study. Three horses were geldings and two were mares (bodyweight 535- 629 kg). All horses were housed in box stalls for the duration of the study. The horses had free access to hay and water, and were fed 1 kg of a high fibre pellet diet (fibre force, Cavalor) twice daily. All horses received an oral anthelmintic (Moxidectin, 400µg/kg) and were vaccinated against

influenza and tetanus approximately 1 month before commencement of the study. This experiment was approved by the local ethical committee on February 3rd 2014 (approval number 2014/183).

Surgical procedure

The day of surgery the horses received acepromazine maleate IM (Placivet, Kela, 0.02 mg/kg) and tetanus prophylaxis SC (Tetanus antitoxin, Intervet, 3000 IU). For the surgical procedure, the horses were sedated with romifidine hydrochloride (Sedivet, Boehringer Ingelheim, 80 µg/kg) and morphine hydrochloride IV (Morphine HCl, Sterop, 100 µg/kg), followed by induction with ketamine hydrochloride (Anesketin, Dechra, 2.2 mg/kg) and midazolam IV (Dormicum, Roche, 60 µg/kg). Horses were intubated and isoflurane (Isoflo, Abbott) in oxygen/air was used for anaesthetic maintenance.

Both metacarpi were first clipped and then shaved with a razor blade from fetlock to halfway the carpus, and were prepared for aseptic surgery. On the dorsomedial aspect of each metacarpus, two circular wounds of 3.5 cm diameter, 4 cm apart, were created by removing skin and subcutis with a scalpel (Fig. 1). In the central part of each wound, the periosteum was removed over a circular area of 2 cm diameter and the underlying bone was curreted 15 times in a dorsopalmar and 15 times in a proximodistal direction (Fig. 2). Sterile templates were used to standardise wound dimensions.

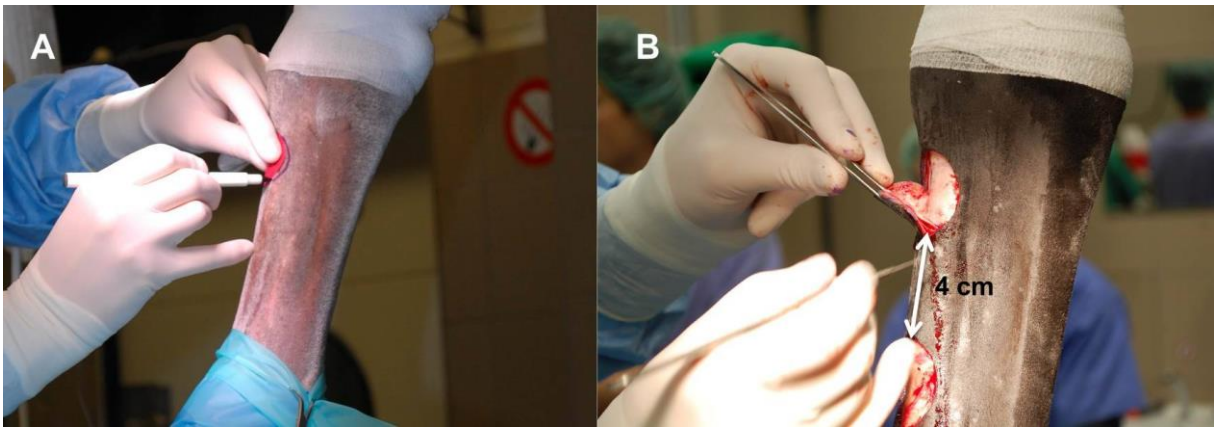

Figure 1. (A) On the dorsomedial aspect of each metacarpus two circular wounds of 3.5 cm diameter were created using a sterile template. (B) The skin and subcutis were removed with a scalpel and the wounds were spaced 4 cm apart.

Immediately after surgery, the wounds were covered with a sterile non-adherent, absorbent dressing (Zorbopad™, Millpledge veterinary), which was held in place by an elastic retention dressing (glatt Lux™, Mai med). On top, a standard limb bandage with cotton wool as a

secondary layer and an elastic bandage (Ideaflex™, Hartmann) as a third layer was applied. The horses were then recovered from general anaesthesia.

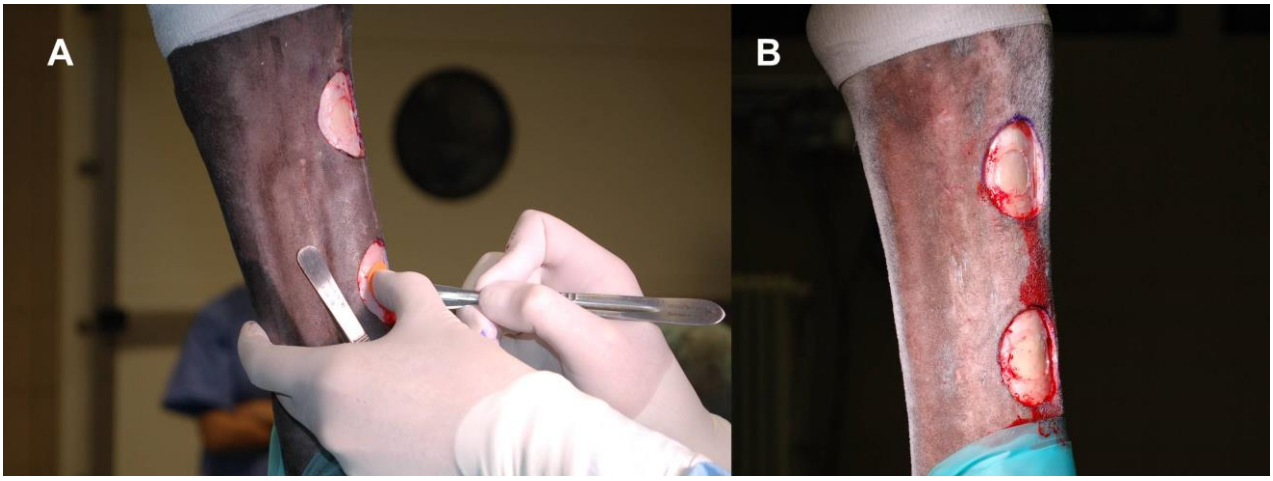

Figure 2. (A) In the central part of each wound, the periosteum was removed over a circular area of 2 cm diameter using a sterile template. (B) The underlying bone was curreted 15 times in a dorsopalmar and 15 times in a proximodistal direction.

Wound management

The day after surgery the bandages of both limbs were removed and one limb was randomly assigned to NPWT using a standard polyurethane foam (V.A.C. GranuFoam™, KCI medical) as primary dressing, while the contralateral limb was assigned to calcium alginate dressings (Kendall) as a reference treatment (REF).

Before application of the treatments, all measurements were performed as described under the subsequent headings. For the REF limb, the calcium alginate dressing was cut into a 10 cm by 10 cm square per wound and moistened with 20 mL of sterile saline solution (0.9 % NaCl). The moistened calcium alginate dressing was then moulded to the wound to minimize overlap with the surrounding skin. The primary dressing was covered with an elastic retention dressing (glatt Lux™, Mai med) and a standard limb bandage was applied as described earlier. For the NPWT limb, the skin surrounding the wounds was degreased with ether and adhesive spray was applied. Next, the entire dorsomedial aspect of the metacarpus (including the wounds) was covered with an occlusive polyurethane foil (V.A.C. drape™, KCI medical) and sterile scissors were used to excise the foil at the level of the wounds to free them. A standard polyurethane dressing (V.A.C. GranuFoam™, KCI medical) was cut into a spectacle-like shape to fit both wounds with a bridge in between them, and was applied to the wounds (Fig. 3). The dressing was fixed onto the wounds and sealed airtight with the occlusive polyurethane foil (V.A.C. drape™, KCI medical). A 2 cm opening was cut into the occlusive foil over the bridge halfway the two wounds, and a suction pad was applied (Fig. 3).

The suction pad was connected to the canister of the NPWT system (V.A.C. ATS™, KCI medical) and the negative pressure was set at 125 mm Hg in continuous mode. A standard limb bandage was applied over the NPWT, as described for the REF limb. During the NPWT treatment the horses were tethered at both sides of their head to minimize their range of movement and prevent them from lying down. The NPWT system was fixed to the wall at the side of the NPWT treated limb (Fig. 4). The horses had free access to haylage, hay and water during this period and received acepromazine maleate PO (Placivet, Kela, 2ml) four times a day as a mild sedative. The NPWT system and the animals were monitored (e.g. pressure level, leaks and blockades of the system and pain, distress in the animals,…) every hour during the day and every two hours at night.

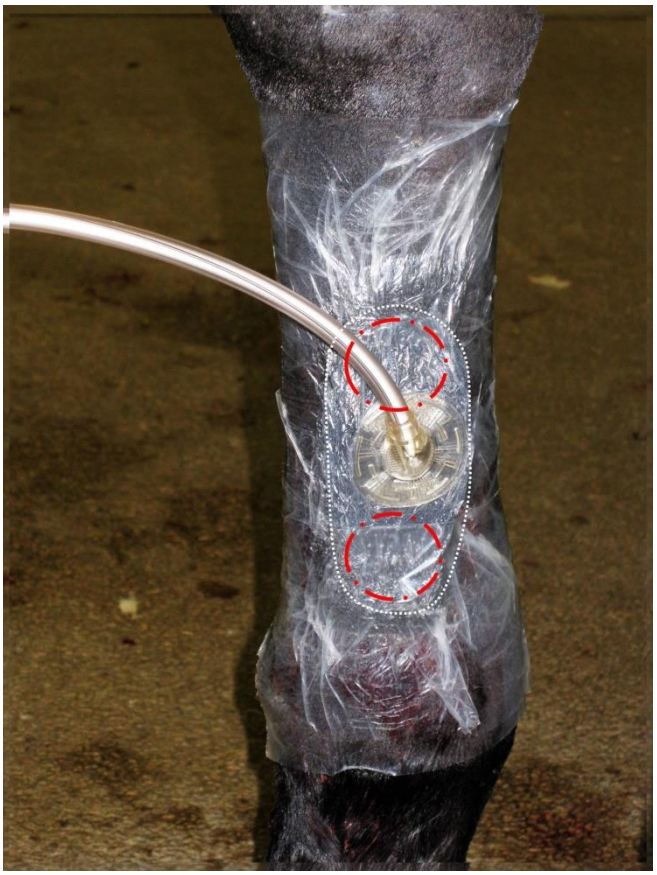

Figure 3: A picture of the negative pressure wound therapy bandage. A standard polyurethane dressing was cut into a spectacle-like shape (white dotted line) to fit both wounds with a bridge in between them, and was applied to the wounds (red dashed circles). The dressing was fixed onto the wounds and sealed airtight with the occlusive polyurethane foil and a 2 cm opening was cut into the occlusive foil over the bridge just halfway the two wounds over which a suction pad was applied.

The NPWT and REF treatments were applied for 9 days with bandage changes every 3 days. Afterwards, both limbs were treated with hydrophilic polyurethane foam dressings (Kendall) with bandage changes every 5 to 7 days. The wounds were monitored for a period of 71 days. The proximal wounds on both limbs were used for the non-invasive measurements, while the distal wounds were biopsied at regular time points to follow up the quality of healing and the inflammatory response. If the granulation tissue exceeded the surrounding skin by 2 to 3 mm during a bandage change, the excess tissue was resected with a scalpel blade after having obtained all the samples of that bandage change. For this procedure, the horses were

sedated with detomidine hydrochloride (Detogesic, Zoetis, 10 µg/kg) and butorphanol tartrate IV (Torbugesic, Zoetis, 10 µg/kg).

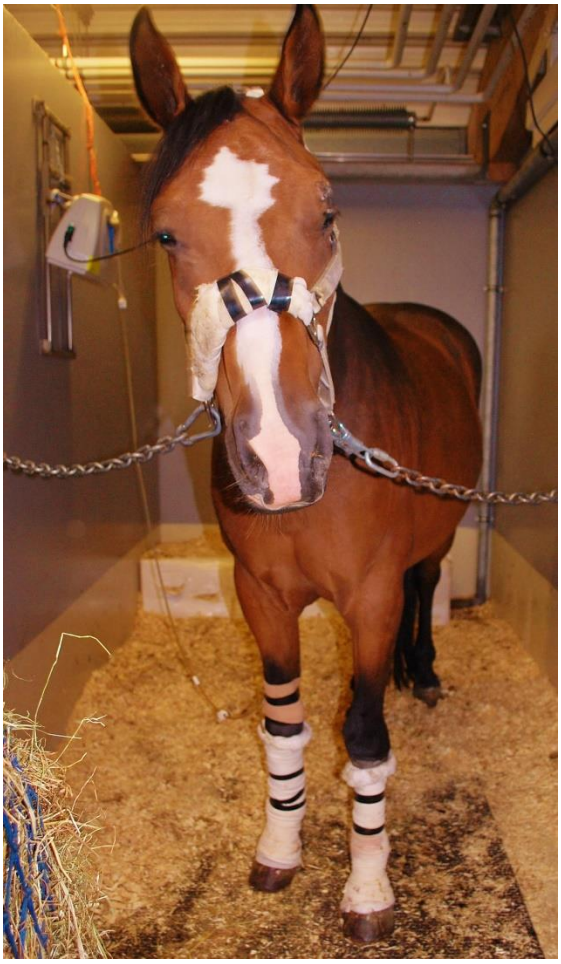

Figure 4. Animal housing

During the negative pressure wound therapy treatment (NPWT) the horses were housed in a narrow stable, tethered at both sides of their head to minimize their range of movement and prevent them from lying down. The NPWT system was fixed to the wall at the side of the NPWT treated wound.

Non-invasive measurements

During the bandage changes, on days 0, 3, 6, 9, 14, 19, 24, 29, 36, 43, 50, 57, 64 and 71, the wounds were first clinically assessed using a checklist (table 1). Next, the wounds were cleaned using sterile gauzes moistened with sterile saline solution (0.9 % NaCl), and swabs for quantitative bacteriology were taken using the Levine technique (Levine et al., 1976). Briefly, a rayon tipped swab was rotated 360° between thumb and index fingers centrally over 1 cm² of the wound during 5 sec. The swab was placed in a container without medium and labelled with the horse's identification, the date, number of days post-surgery, and wound reference. The swabs were stored at 4°C for a maximum of 4 hours before they were processed in the laboratory. To evaluate limb swelling, the circumference of the limb was measured immediately distal to the proximal wound using a measuring tape. Blood flow was evaluated indirectly using a thermographic camera (ThermaCam E2, FLIR Systems) as described by Celeste et al. (2011). Briefly, all thermographic images were taken in the same

room, of which the humidity and ambient temperature were documented to standardize the measurements afterwards using software (FLIR tools, FLIR systems). All images were taken perpendicular to the wound at 40 cm distance approximately 15 minutes after removal of the bandages. Wound dimensions (surface area, maximum depth and volume) were measured using a laser beam camera (SilhouetteStarTM, ARANZ Medical Ltd) as previously described (Van Hecke et al., 2015).

Table 1. The checklist used to evaluate the wounds for signs of infection.

If the sign listed was present in the wound, the box next to the description was checked off. The rows with three check boxes distinguish between the different signs listed, while in the rows with one checkbox, a check was placed if either one of the described signs was present.

Exudate (oozed through the third layer of the bandage) clear / sanguineous / purulent			
Red granulation tissue (beefy aspect)			
Yellow necrosis/slough/discoloration of the granulation tissue			
Black necrosis/slough/discoloration of the granulation tissue			
Exuberant granulation tissue			
Oedematous granulation tissue (glassy, shiny aspect)			
Friable granulation tissue (easily bleeding when probing the surface and base of the wound)			
Bone visible or felt with a probe			
Oedema around the wound/ part of the limb /entire limb			
Unpleasant odour			
Pain (it is difficult to touch the wound even when the horse is sedated)			

Invasive measurements

On days 3, 6, 9, 19 and 29, an 8 mm punch biopsy was taken along the wound edge of the distal wounds of both limbs. Horses were sedated with detomidine hydrochloride (Detogesic, Zoetis, 10 µg/kg) and butorphanol tartrate IV (Torbugesic, Zoetis, 10 µg/kg) and about 2 mm of skin and/or epithelial border and 6 mm of granulation tissue were included in the biopsy. The location of the biopsy was alternated following a star shaped pattern (Fig. 5), of which the first biopsy site was randomly assigned. The biopsies were placed in a sterile container and labelled with the horse's identification, number of days post-surgery and wound reference. The biopsies were stored at 4°C for a maximum of 4 hours before they were processed in the laboratory. Half of the biopsy was used for histology to follow up the

inflammatory response and the other half was used for growth factor analysis of transforming growth factor β1 (TGF-β1) and transforming growth factor β3 (TGF-β3).

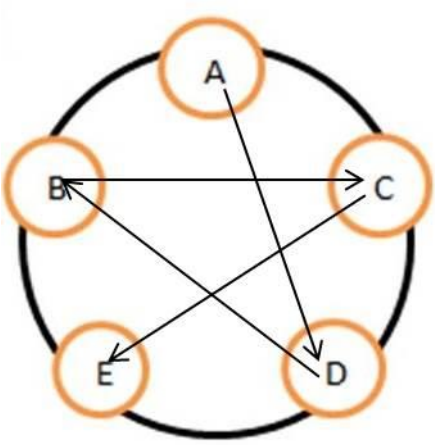

Figure 5. Schematic overview of biopsy scheme
On days 3, 6, 9, 19 and 29, an 8 mm punch biopsy was taken along the wound edge of the distal wounds of both limbs. About 2 mm of skin and/or epithelial border and 6 mm of granulation tissue were included in the biopsy. The location of the biopsy was alternated following a star shaped pattern, of which the first biopsy site was randomly assigned.

Quantitative bacteriology

The tip of the swab was cut off with sterile scissors and put into a 1.5 mL Eppendorf tube filled with 1mL of sterile phosphate buffered saline (PBS). The tip was vortexed during 30 seconds and then serially diluted 10-fold in PBS. Five 10µL aliquots of each dilution were spot-plated onto a Columbia agar supplemented with 5 % sheep blood (Oxoid). The Columbia agar plates were incubated in a 5 % CO2 enriched atmosphere at 37°C for 24 hours, after which the colony-forming units (CFU) were counted. The number of CFU per swab was calculated taking the dilution into account.

Growth factor analysis

Tissue samples were aseptically weighed and processed using a standard mammalian cell lysis kit (Sigma Aldrich) containing a protease inhibitor cocktail. Briefly, the biopsy was washed twice in chilled (4°C) sterile PBS and finely diced before adding it to the cell lysis buffer (1 mL for 5-20 mg of tissue). The biopsy was incubated in the buffer for 30 minutes on an orbital shaker in a chilled styrofoam box. Next, the biopsy was homogenized using a polytron homogeniser (PT1200 E, Kinematica) and centrifuged in a chilled centrifuge (4 °C, Heraeus multifuge 1SR, Thermo scientific) for 10 minutes at 5000 g. The supernatant was aliquoted and frozen at -20 °C.

For the TGF-β1 and TGF-β3 analysis an equine enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturers' guidelines (Eq TGFb1 kit, Genorise, for TGF-β1 and SEB949Eq, Cloud-Clone corp., for TGF-β3). All samples were thawed before

processing. The sensitivity of the TGF-β1 and TGF-β3 assay was 8 pg/mL and 6.3 pg/mL respectively.

Histology

Tissue samples were fixed in 4% formaldehyde, embedded in paraffin and cut into 4 µm slices. On each sample a Hematoxylin and eosin (HE) and Masson trichrome stain was performed. Microscopic evaluation of all samples was performed by the first author, who was blinded from treatments. The samples were scored based on the scoring system described by Demaria et al. (2011). Briefly, neutrophilic infiltration, tissue oedema, extent of haemorrhage and tissue necrosis were scored from 0-3. The scores of these 4 parameters were added to obtain the histologic acute inflammation score (HAIS; range 0-12). To assess the quality of the granulation tissue, the fibroblast proliferation, collagen density and collagen orientation were scored from 0-3. Since collagen orientation was not scored by Demaria et al. (2011), the used scoring values of the collagen orientation are described here: 0 = absence of collagen, 1 = random orientation of the collagen fibres, 2= majority of the collagen fibres perpendicular to the wound surface, 3 = majority of the collagen fibres parallel to the wound surface. The scores of these parameters were added to a histologic repair score (HRS; range 0-9).

Additionally, the samples were stained using immunohistochemistry for the detection of B-cells (polyclonal rabbit anti-CD20, Thermo Scientific, 1:100), T-cells (polyclonal rabbit anti-human CD3, Dako, 1:100), macrophages (monoclonal mouse antibody MAC387, Abcam, 1:100), endothelium (rabbit anti-human vonWillebrand Factor, Dako, 1:3200) and myofibroblasts (monoclonal mouse anti-human Smooth muscle actin clone 1A4, Dako, 1:200). Immunolabeling was achieved using a highly sensitive horseradish peroxidase mouse or rabbit diaminobenzidine kit (Envision DAB+ kit, Dako) in an auto-immunostainer (Dako). This kit also blocked endogenous peroxidase. Positive staining was confirmed on light microscopy, and the area percentages (ratio positive stained cells on entire high power field) of three to five (depending on the size of the biopsy) photographed areas (2 or 1 in the superficial layer of biopsy, 1 in the middle layer and 2 or 1 in the deep layer) were calculated per section using LAS V4.0 software (LEICA Microsystems).

Statistical analysis

Statistical analysis was performed using SPSS statistics 20 (IBM). The clinical assessment parameters were analysed over the entire follow up period using a generalized linear model with a binomial probability distribution and a logit link function. The model was corrected for dependent observations by defining horse as a subject effect and leg and time as within

subject effects. Clinical parameters were used as dependent variables and the respective treatments as independent variables. For the other non-invasive measurements, the follow-up period was divided into 3 time periods, because there were too little observations to analyse each sample point separately: the treatment period from day 0 until 9, the early period after treatment from day 14 until 29 and late period after treatment including day 36 until 71. An analysis of variance for repeated measures was performed with time period and treatment and their interactions as crossed fixed factors. For the analysis of the histological data, a generalized linear model with multinomial probability distribution and cumulative logit link function was used. The model was corrected for dependent observations by defining horse as a subject effect and leg and time as within subject effects. The scores for the different parameters were used as dependent variable and treatment as independent variable. The area percentages of the immunohistological data were analysed using a generalized linear model with a binomial distribution and a logit link function. Again, the model was corrected for dependent observations by including horse as subject effect and leg and time as within subject effects. The area percentages were used a dependent variable and the treatment as independent variable. The histological and immunohistological data were analysed for all the time points combined (days 3, 6, 9, 19 and 29) to estimate the overall effect. For the growth factor analysis, an analysis of variance for repeated measures was performed with time and treatment and their interactions as crossed fixed factors. A P-value of < 0.05 was considered to be statistically significant. All model assumptions were checked and met.

Results

Wound dimensions

The wound area of NPWT wounds was significantly smaller compared to REF wounds during the treatment period (mean difference of 19% $P = 0.005$) and the early period after treatment (mean difference of 9% $P = 0.003$) (Fig. 6). This was no longer the case in the late period after treatment. As expected, for both treatments the wound area was significantly larger during the treatment period compared to the early period and late after treatment and in the early period compared to the late period after treatment ($P \leq 0.001$) (Fig. 6).

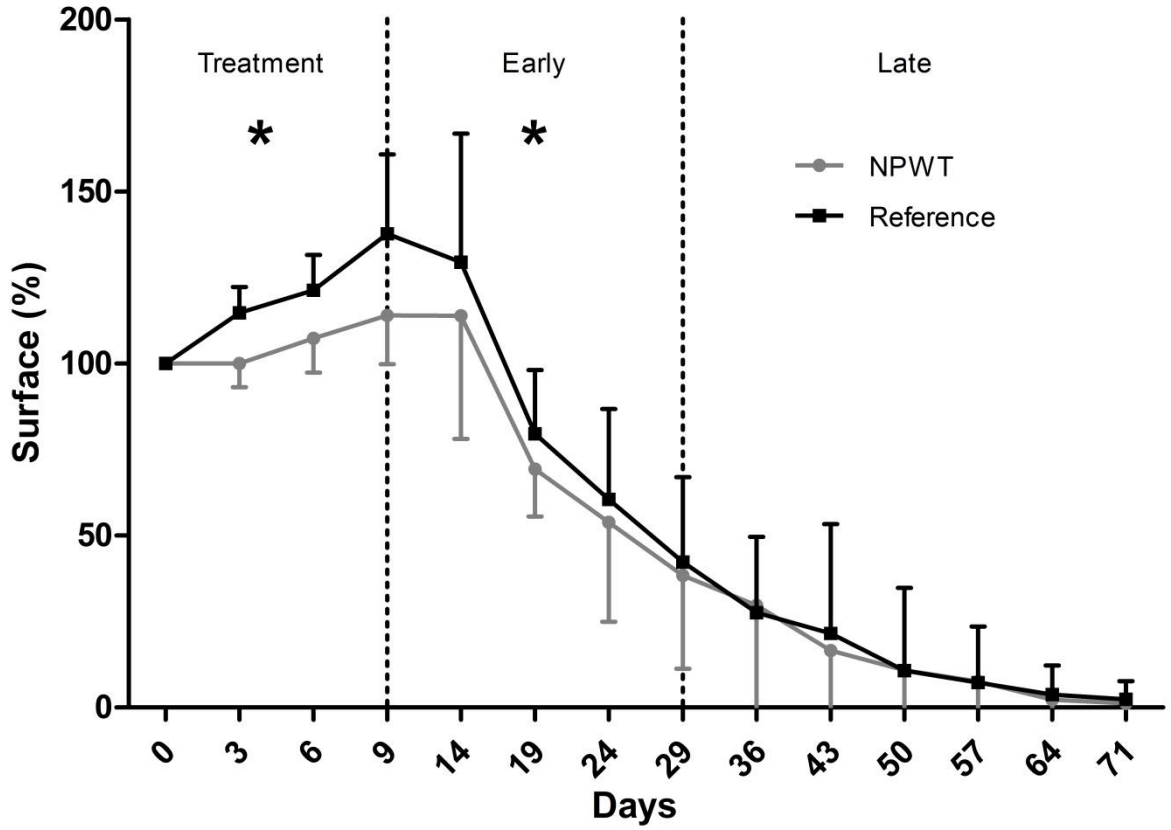

Figure 6. Evolution of wound surface area over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 9 (Treatment), the early period after treatment from day 14 until 29 (Early) and late period after treatment including day 36 until 71 (Late). *: the mean surface area for this time period differed significantly between the treatment groups ($P < 0.05$). NPWT: negative pressure wound therapy. Reference: Ca-alginate dressings.

The variance of the data on wound volume was too large to allow comparison between treatments and over time.

The maximum depth of NPWT wounds was significantly larger compared to REF wounds during the treatment period (mean difference of 78% $P = 0.007$) and the early period after treatment (mean difference of 87% $P = 0.029$) (Fig. 7). In the late period after treatment there was no longer a significant difference. For both treatments, the maximal wound depth was significantly larger during the treatment period compared to the late period after treatment (Fig. 7).

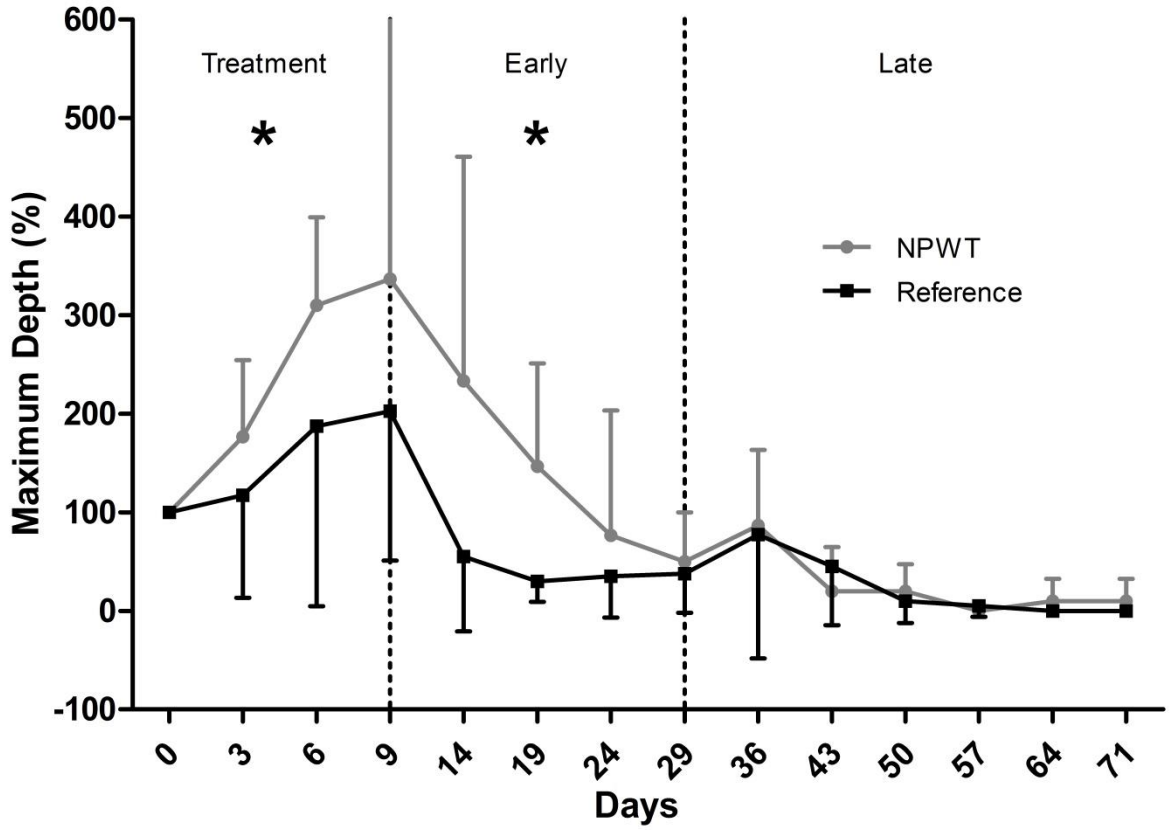

Figure 7. Evolution of maximum wound depth over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 9 (Treatment), the early period after treatment from day 14 until 29 (Early) and late period after treatment including day 36 until 71 (Late). *: the mean maximum wound depth for this time period differed significantly between the treatment groups ($P < 0.05$). NPWT: negative pressure wound therapy. Reference: Ca-alginate dressings.

Clinical assessment

On clinical assessment, REF wounds were found to have a significantly higher chance to present oedematous granulation tissue ($P = 0.01$) and an unpleasant odour ($P < 0.001$) compared to the NPWT wounds. Exuberant granulation tissue was trimmed 14 times in REF wounds and 13 times in NPWT wounds and was mostly present in the early period after treatment. There was no significant difference in the presence of exuberant granulation tissue between the two treatments.

Histology

The REF wounds had a significantly higher (P = 0.044) histological score for acute inflammation compared to the NPWT wounds. When examining the different parameters of the HAIS, the REF wounds had a significantly higher neutrophilic infiltration compared to the NPWT wounds (P < 0.001). For the parameters tissue oedema, extent of haemorrhage and tissue necrosis, no significant difference was seen between the NPWT and REF wounds. However, in the NPWT-treated wounds significantly (P = 0.008) more macrophages were present compared to the REF wounds. The presence of B-cells, T-cells, myofibroblasts and neovascularisation was not significantly different between the NPWT and REF wounds. There was also no significant difference in HRS, nor in its different parameters between the REF and the NPWT wounds.

Bacterial load, limb swelling and wound temperature

There was no significant difference in bacterial load between the different treatments. Within a treatment, the bacterial load was also not significantly different over time.

During the treatment period, NPWT limbs were significantly more swollen compared to REF limbs (P = 0.036). This was not the case during the early and late period. In REF limbs, there was no significant difference in swelling over time, while for NPWT limbs, the swelling was significantly more pronounced in the treatment period compared to the late period after treatment (P = 0.003).

There was no significant difference in temperature, thus indirectly blood flow, between treatments. However, for both treatments, temperature was higher during the treatment period compared to the early period after treatment (P = 0.018) (Fig. 8).

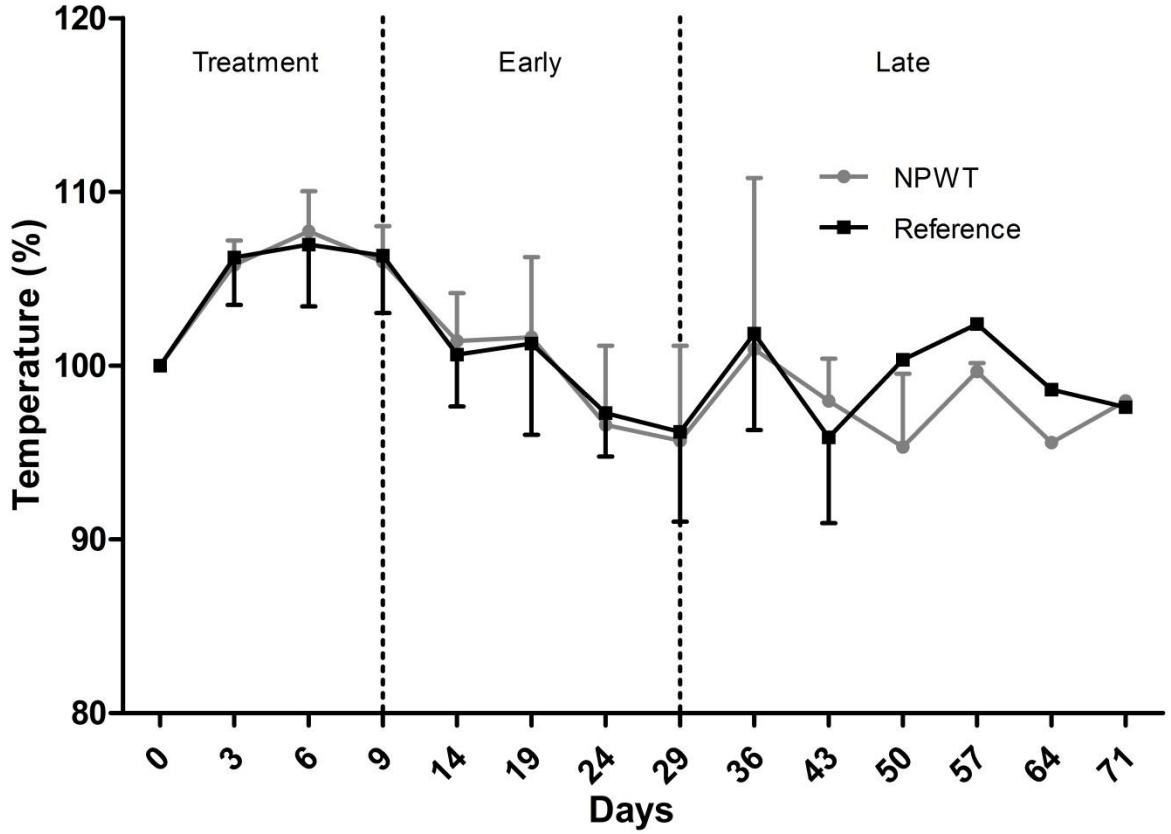

Figure 8: Evolution of wound temperature (an indirect measure for blood flow) over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 9 (Treatment), the early period after treatment from day 14 until 29 (Early) and late period after treatment including day 36 until 71 (Late). NPWT: negative pressure wound therapy. Reference: Ca-alginate dressings.

Growth factor analysis

There was no significant difference in TGF-β1 concentration between NPWT and REF wounds. However, in both treatment groups, TGF-β1 increased steadily over time and at day 29, the TGF-β1 concentrations were significantly higher compared to day 3.

There was no significant difference in TGF-β3 concentration between NPWT and REF wounds or within treatment groups between time points.

Discussion

The aim of the study was to investigate the clinical relevance of NPWT for the treatment of acute non-inoculated distal limb wounds in horses. Therefore, we designed a model simulating real-life traumatic equine limb wounds, which are relatively large, round shaped

when healing by second intention and often with involvement of exposed cortical bone. This is in contrast to the conventional wound models used in most equine studies, which are relatively small (1-7cm²) and rectangular in shape (Wilmink et al., 1999; Theoret et al., 2001; Theoret et al., 2002; Monteiro et al., 2009; Bischofberger et al., 2011; Bischofberger et al., 2013; Broeckx et al., 2015; Bischofberger et al., 2016). The circular shape of our wound model also provided a better fit with the suction pad of the NPWT system. Tracey et al. (2014) also used a wound model with a large surface area (12 cm²), but the wounds were rectangles and had a very narrow shape (2 cm x 6 cm). This type of wound is likely to heal faster than a traumatic wound because of the limited wound retraction in proximodistal direction and the small distance between the vertical wound edges, making this wound model less realistic. Similar to other equine studies, the wounds in our model were located medially to the common digital extensor tendon to prevent wound healing complications by the movement of the tendon in the wound bed (Wilmink et al., 1999; Theoret et al., 2001; Theoret et al., 2002; Monteiro et al., 2009; Bischofberger et al., 2011; Tracey et al., 2014; Bischofberger et al., 2016). Our wound model was well tolerated by the horses, despite the large wound surface area. The horses did not show any signs of discomfort or pain in the stables and were not lame. During the first bandage changes (day 0-9) they showed some reaction during wound cleaning, but this was resolved by sedating the horses.

In our study, repeated biopsies were taken along the wound edge to provide insight in the histological evolution of one wound. In contrast, other equine studies use multiple small wounds which are only biopsied once, assuming equal wound healing in all wounds (Theoret et al., 2001; Theoret et al., 2002; Monteiro et al., 2009; Bischofberger et al., 2011). To minimize the influence of the repeated biopsies on each other in our wound model, every site was only biopsied once and the distance between the sites was maximized by using a star shape pattern (Fig. 5).

The authors wanted to compare NPWT to the standard of care in their institutions, which is based on research and clinical evidence (Stashak, 2008, Wilmink, 2008), and to investigate the added value of NPWT to this standard. Ca-alginates are used for the first period of healing because of horses' initial weak inflammatory response in distal limb wounds. These dressings stimulate inflammation and induce a faster formation of healthy granulation tissue (Wilmink et al., 1999; Hendrickson, 2002; Wilmink, 2008). The stimulation of granulation tissue formation and cell proliferation is also one of the main reasons NPWT is used in humans for treating chronic wounds or wounds with massive tissue loss (Mouës et al., 2011). Placing a NPWT bandage in a horse is often associated with practical difficulties, and is considerably costlier than calcium alginate dressings. For example, in our institution NPWT costs an owner daily 45 EUR, while treatment with calcium alginate dressings costs 5.5 EUR

daily, both without wages. Therefore, NPWT should significantly reduce the overall healing time, reduce complications or improve the quality of the scar tissue making it is less susceptible to reinjury, to be cost effective. In our study, NPWT-treated wounds retracted less compared to REF wounds during the treatment period (day 0-9). The surface area of NPWT-treated wounds was still smaller than REF wounds during the early period after treatment (day 14-29), but during the late period after treatment (day 36-71) the healing of REF wounds caught up with NPWT-treated wounds and there were no longer significant differences in wound area. The maximum depth of NPWT-treated wounds was larger during the treatment period and early period after treatment compared to REF wounds, which indicates that the calcium alginate therapy induced a more rapid formation of granulation tissue compared to the NPWT. Additionally, the larger maximum depth of NPWT-treated wounds in the treatment period could also be caused by deformation of the wound by the suction of NPWT. The suction force moves the wound edges somewhat centripetally, but perhaps also somewhat higher thus artificially enlarging the maximum depth of the wounds. However, during the late period after treatment, the maximum depth of both NPWT-treated wounds and REF wounds was similar. Thus, no difference was seen in the overall healing time between the two treatments.

The less pronounced retraction in NPWT wounds is probably due to mechanical influence of NPWT: NPWT physically keeps the wound edges in place by a combination of the negative pressure applied to the wound bed and the adhesive foil applied over the wound. This theory is further reinforced by the absence of a significant difference in myofibroblasts or collagen orientation between the two treatments.

The histological results showed a significantly higher HAIS for REF wounds with a higher neutrophilic influx in the wound, but the amount of tissue oedema on histology was not significantly different even though the clinical parameter ‘presence of oedematous granulation tissue’ was elevated for REF wounds. Therefore, the observed differences in the subjective clinical parameters of inflammation should be carefully interpreted.

The bacterial load was not significantly different between the two treatments, so this could not explain the difference seen in the inflammatory response on histology or the difference in odour. Therefore, the differences in infiltration of inflammatory cells will probably be an expression of different immunomodulating effects of the treatments. A possible explanation for the higher prevalence of an unpleasant odour in REF wounds is the intrinsic smell of the calcium alginate dressings when they absorb fluid.

Interestingly, NPWT limbs were significantly more swollen (measured with tape measure) in the treatment period (day 0-9) than the REF limbs, while NPWT is said in the literature to

reduce oedema (Kilpadi et al., 2006). However, Mouës et al. (2011) already mentioned the low value of the studies which investigated the effect of NPWT on oedema, because of their low inclusion numbers. In contrast to the reduction of oedema, the stimulating effect of NPWT on blood flow is considered to be a proven mechanism of action of NPWT in men (Birke-Sorensen et al., 2011; Mouës et al., 2011,). However, the blood flow in the wounds of both NPWT and REF, indirectly determined by measuring temperature (Celeste et al., 2011), was not significantly different. Additionally, no significant difference in neovascularisation between the REF and NPWT wounds was seen during histological examination. Apparently, the extent of stimulation of blood flow and neovascularisation was similar for NPWT and REF-treated wounds.

In our study, TGF-β1 concentrations increased steadily over time, with a significant increase on day 29 compared to day 3. This temporal distribution of TGF-β1 is different from three earlier studies on TGF-β1 concentrations in untreated equine distal limbs (Theoret et al., 2001; Theoret et al., 2002; Van Den Boom et al., 2002). The studies of Theoret et al. (2001; 2002) reported a peak of TGF-β1 in the early inflammatory phase (24 hours after wounding) followed by a rather stable, but elevated concentration for the following 14 days. Van Den Boom et al. (2002) also demonstrated a peak of TGF-β one day after wounding, with a second peak at day seven after which the TGF-β levels remained elevated during the two week study period. The increasing concentration in our study could be induced by the chosen wound treatments, which are both known to stimulate fibroplasia (Stashak, 2008; Mouës et al.,2011). Other influencing factors could be the use of a human ELISA kit from a different manufacturer in other studies (Theoret et al., 2001; Theoret et al., 2002) or the investigation of overall TGF-β concentrations instead of only TGF-β1 (Van Den Boom, 2002).

When evaluating the wounds histologically, a HAIS with a higher neutrophilic infiltration was seen for REF wounds compared to NPWT wounds. Although neutrophils are the first line of defenses in wound healing (Theoret et al., 2008b), their higher infiltration in REF wounds did not translate into a lower bacterial load compared to NPWT wounds, nor in a faster overall wound healing. A higher infiltration of macrophages was observed in NPWT-treated wounds compared to REF wounds. Macrophages appear in the wound bed after the neutrophils and have an indispensable role in wound healing. They are necessary for the initiation and propagation of the fibroplasia (Theoret, 2008b). However, in this study the higher infiltration of the macrophages did not lead to a better HRS or more rapid granulation tissue formation for NPWT wounds. Therefore, both treatments stimulated the inflammatory response, be it on a different level, but neither treatment gave a faster overall wound healing compared to the other. These findings concur with more recent findings on the role of leukocytes in wound healing. Both neutrophils and macrophages play an important, complex and intertwining role

in wound healing, partially capable of taken over each other's role to some extent. However, the complex interactions between these cells and the wound environment is still not fully elucidated (Koh and DiPietro, 2011; Wilgus et al., 2013).

In veterinary medicine, only two other controlled trials have been performed on NPWT (Demaria et al., 2011; Stanley et al., 2013). Demaria et al. (2011) tested the effect of NPWT on acute excisional wounds in dogs healing by second intention, while Stanley et al. (2013) investigated the effect of NPWT on free full-thickness skin grafts in dogs. Demaria et al. (2011) found that NPWT-treated wounds retracted more during the first 7 days and contracted less afterwards, epithelialization was retarded, the bacterial load was higher on day 7 and that the HAIS was higher on day 3 but lower in day 7. In our study we found the opposite: NPWT-treated wounds retracted less the first 9 days, were still smaller in the early period after treatment, the bacterial load did not differ between the two treatments and the HAIS was higher in REF wounds. However, the study of Demaria et al. (2011) and our study are difficult to compare because of the differences in species and study design. Demaria et al. (2011) did not consistently use the same primary dressing for all the NPWT-treated wounds, they used a different control/reference dressing (hydrophilic semi-occlusive polyurethane foam), and they applied NPWT for the entire follow up period (21 days). In our study, we applied NPWT for 9 days until most of the wounds were fully granulated. This would also be course of action in a clinical patient.

The number of horses used for this study was limited because of practical and ethical considerations. Despite the low number, several significant differences between the treatments were observed. However, for some parameters with a large variance (e.g. wound volume) this number was too low.

In the future, it would be interesting to make this wound model even more compliant to traumatic wounds by inoculating the wounds. This was not performed in this study because the authors wanted to test the feasibility and tolerability of this model first.

The information of this study can also be interesting for human medicine, as equine distal limb wounds can act as a model of chronic wound healing in men (Theoret and Wilmink; 2013; Wilmink, 2014) and randomized experimental studies cannot be performed.

Conclusion

No real advantage could be detected in the use of NPWT over calcium alginate dressings for acute non-inoculated equine distal limb wounds healing by second intention, especially when considering the costs of NPWT and practical difficulties in applying it.

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THE EFFECT OF NEGATIVE PRESSURE WOUND THERAPY ON SECOND INTENTION HEALING IN CONTAMINATED EQUINE DISTAL LIMB WOUNDS: A RANDOMIZED CONTROLLED EXPERIMENTAL STUDY

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Summary

The goal of this study was to assess the added value of Negative Pressure Wound Therapy (NPWT) on second intention healing of contaminated equine distal limb wounds compared to silver Ca-alginate dressings. Two circular 3.5 cm diameter wounds were created on both metacarpi in 5 horses. Each wound was inoculated with two common equine wound pathogens: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In the first 6 days, one limb was treated with NPWT, while the contralateral limb was treated with silver calcium alginate dressings (reference). From day 6 to 71, both limbs were kept under bandage with hydrophilic polyurethane foam dressings. Over the course of healing, wound dimensions were measured, swabs were taken for quantitative bacteriology and blood flow was indirectly measured using a thermographic camera. On 5 time points, an 8 mm punch biopsy was taken for growth factor analysis, histological scoring of inflammation and repair, as well as immunohistochemical demonstration of B-cells, T-cells, macrophages, endothelium and myofibroblasts.

No significant difference between the two treatments could be detected for any of the wound dimensions (surface area, maximum depth and wound volume). There were also no significant differences in bacterial load, wound temperature (hence blood flow) and limb swelling between NPWT and reference wounds. There was no significant difference in histological inflammation and repair scores, nor in the presence of B-cells, T-cells, macrophages, myofibroblasts and neovascularisation. The concentration of transforming growth factors β1 and β3 was not significantly different between the two treatments.

Based on the results, no real advantage could be detected in the use of NPWT over silver Ca-alginate dressings for treatment of contaminated distal limb wounds in horses.

Introduction

Traumatic wounds are very common in horses and are a cause of noteworthy morbidity and mortality (Theoret et al., 2016). These wounds are often left open to heal by second intention because of massive tissue loss and high skin tension (Theoret, 2008). Moreover, when traumatic wounds are localized at the distal limb, they display an impeded wound healing reminiscent of chronic non-healing wounds in humans such as pressure ulcers and diabetic ulcers (Theoret, 2008; Wilmink, J.M., 2014). During second intention healing, the inflammatory response of equine distal limb wounds displays a slow onset, does not show an effective peak and persists over time, leading to chronic inflammation (Wilmink, 2008). Additionally, traumatic wounds at the distal limb are often heavily contaminated with micro-organisms (Theoret, 2008). This contamination can evolve towards an infection depending on several factors, such as the virulence of the micro-organisms, the number of micro-organisms, the nature of the wound, and the host's immune response (Sibbald et al., 2006). An ideal wound treatment should assess the chronic inflammation in distal limb wounds and decrease bacterial contamination, while stimulating the formation of healthy granulation tissue. Several studies have been performed seeking such a therapy, but with varying success (Monteiro et al., 2009; Bischofberger et al., 2011; Lepage et al., 2012; Bischofberger et al., 2013, Kelleher et al., 2015; Broeckx et al., 2015; Bischofberger et al., 2016). To the authors' knowledge, a wound treatment which would potentially address all the issues of equine distal limb wound healing has not been found yet.

Negative pressure wound therapy (NPWT) is a treatment which is well-established and frequently used in human medicine for chronic wounds (Vig et al., 2011). This technique applies a continuous or intermittent negative pressure to the wound bed to expedite wound healing (Morykwas et al., 1997). NPWT has been shown in men to increase local blood supply of the wound, stimulate angiogenesis and granulation tissue formation, reduce wound surface area and remove excess exudate, so it could aid the healing of distal limb wounds in the horse. However, the effect of NPWT on wound contamination or infection with different bacterial species is still controversial (Morykwas et al., 1997; Mouës et al., 2004; Mouës et al., 2011; Patmo et al., 2014). Additionally, studies on the antibacterial effect of NPWT mainly use a standard polyurethane foam (Morykwas et al., 1997; Mouës et al., 2004; Weed et al., 2004; Braakenburg et al., 2006), while a silver impregnated polyurethane foam has been specially developed to treat contaminated wounds (Payne and Ambrosio, 2009). In veterinary medicine, studies on the use of NPWT in horses are scarce and mainly entail case reports (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Jordana et al., 2011). To the authors' knowledge, research on the influence of NPWT on contaminated equine distal limb wounds is lacking.

The aim of this study was to assess the added value of NPWT for the treatment of second intention healing of contaminated distal limb wounds of horses over silver Ca-alginate dressings. An inoculated wound model was used to approach real life contaminated wounds. The hypothesis was that NPWT would result in a faster decrease in wound dimensions, would increase the inflammatory response, improve local blood flow and decrease bacterial load compared to silver Ca-alginate dressings.

Materials and methods

Horses

Five warmblood horses (8-17 years of age) with both metacarpi free of scars were used in this study. Three horses were geldings and two were mares (bodyweight 560- 648 kg). All horses were housed in box stalls for the duration of the study. The horses had free access to hay and water, and were fed 1 kg of a high fibre pellet diet (fibre force, Cavalor) twice daily. All horses received an oral anthelmintic (Moxidectin, 400µg/kg) and were vaccinated against influenza and tetanus approximately 1 month before commencement of the study. This experiment was approved by the local ethical committee on 7 October 2015 (approval number 2015/104).

Surgical procedure

The day of surgery the horses received acepromazine maleate IM (Placivet, Kela, 0.02 mg/kg) and tetanus prophylaxis SC (Tetanus antitoxin, Intervet, 3000 IU). For the surgical procedure, the horses were sedated with romifidine hydrochloride (Sedivet, Boehringer Ingelheim, 80 µg/kg) and morphine hydrochloride IV (Morphine HCl, Sterop, 100 µg/kg), followed by induction with ketamine hydrochloride (Anesketin, Dechra, 2.2 mg/kg) and midazolam IV (Dormicum, Roche, 60 µg/kg). Horses were intubated and isoflurane (Isoflo, Abbot) in oxygen was used for anaesthetic maintenance.

Wounds were created as described in the previous chapter (chapter 6.1). Briefly, both metacarpi were first clipped, shaved and then prepared for aseptic surgery. On the dorsomedial aspect of each metacarpus, two circular wounds of 3.5 cm diameter were created, 4 cm apart (Fig. 1). In the central part of each wound, the periosteum was removed over a circular area of 2 cm diameter and the underlying bone was curreted. Sterile templates were used to standardise wound dimensions.

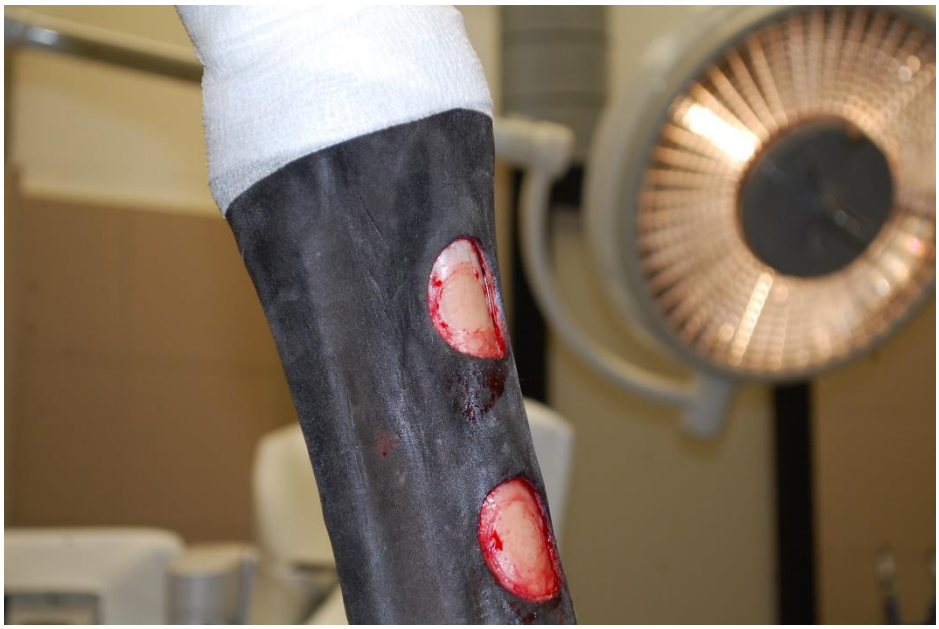

Figure 1. An overview of the surgical procedure.

On the dorsomedial aspect of each metacarpus two circular wounds of 3.5 cm diameter were created, 4 cm apart, by removing the skin and subcutis with a scalpel. In the central part of each wound, the periosteum was removed over a circular area of 2 cm diameter and the underlying bone was curreted.

Immediately after surgery, the wounds were covered with a sterile non-adherent, absorbent dressing (Zorbopad™, Millpledge veterinary), which was held in place by an elastic retention dressing (glatt Lux™, Mai med). On top, a standard limb bandage consisting of cotton wool as secondary layer and an elastic bandage (Ideaflex™, Hartmann) as a third layer was applied. The horses were then recovered from general anaesthesia.

Wound inoculation

The day after surgery, bacterial stock suspensions of two common equine wound pathogens were prepared. A *Staphylococcus aureus* and *Pseudomonas aeruginosa* strain, isolated from equine wound patients, were dissolved in phosphate buffered saline (PBS) (approximately 3 x 10^8 and 2 x 10^8 colony forming units per mL respectively). The bandages of both limbs of the horses were removed and the wounds were cleaned using sterile gauzes moistened with saline solution (0.9 % NaCl). A total of 4 sterile gauzes, cut to the size of the wounds, were prepared per horse. Each gauze was inoculated with 1 mL of the *S. aureus* and 1 mL of the *P. aeruginosa* bacterial stock suspension using an air-displacement micropipette. The inoculated gauzes were positioned on the wound bed, one per wound, and adhesive spray was applied on the surrounding skin. A semipermeable polyurethane foil was used to keep the gauzes in place and to prevent them from desiccating (Fig. 2). A standard limb bandage was applied as described earlier. Twenty-four hours later, the bandages were removed and

the wounds were flushed using sterile saline solution (0.9 % NaCl in a 35 ml syringe with a 19G needle). The inoculation procedure was repeated and a standard limb bandage was applied. The third day after surgery, the bandages of both limbs were removed and the wounds were again flushed with a sterile saline solution (0.9% NaCl in a 35 ml syringe with a 19G needle). The wounds were covered with a sterile non-adherent, absorbent dressing (Zorbopad™, Millpledge veterinary), which was held in place by an elastic retention dressing (glatt Lux™, Mai med). A standard limb bandage was applied as described earlier and the horses were put in a box for 24 hours before commencement of the treatments.

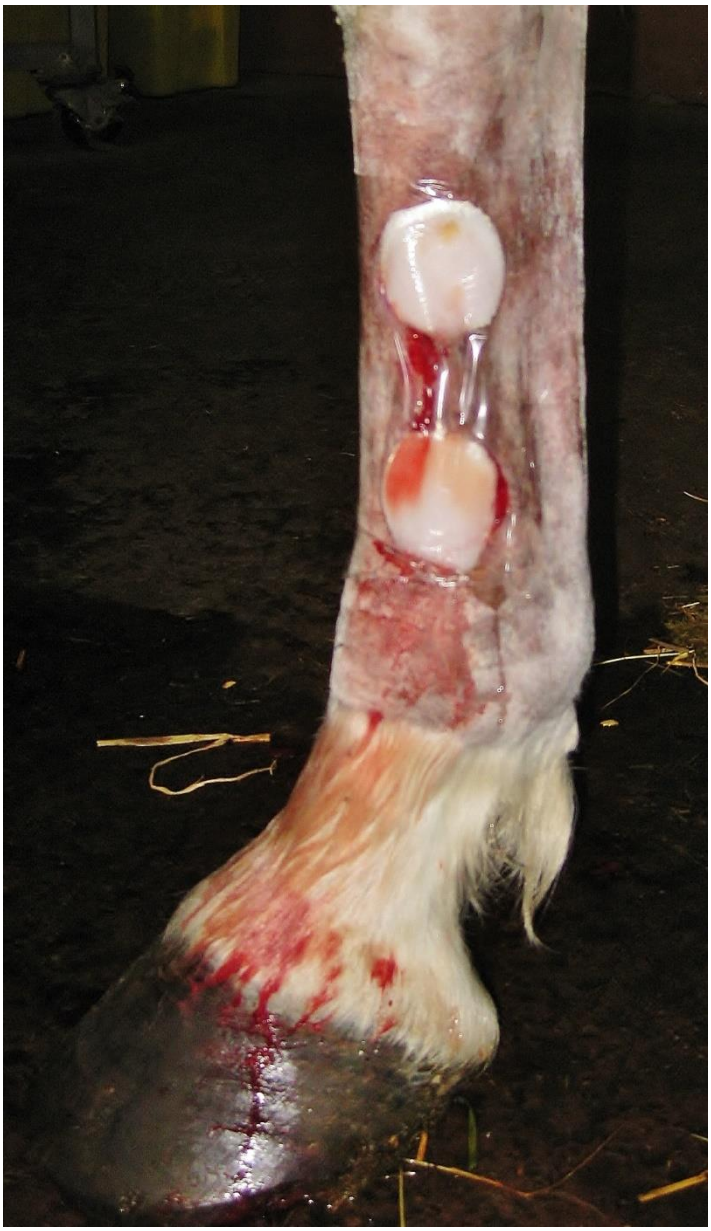

Figure 2. A picture of the inoculation procedure.

The wounds were contaminated with 2 common equine wound pathogens, namely *S. aureus* and *P. aeruginosa*. Sterile gauzes were prepared and each gauze was contaminated with one mL of the *S. aureus* and one mL of the *P. aeruginosa* bacterial stock solution using an air-displacement micropipette. The contaminated gauzes were positioned on top of the wounds, one per wound, and adhesive spray was applied on the surrounding skin. A semipermeable polyurethane foil was used to keep the gauzes in place and to prevent the gauzes from desiccating.

Wound management

After the inoculation protocol (day 4 after surgery), the bandages of both limbs were removed and one limb was randomly assigned to NPWT using a silver impregnated polyurethane foam (V.A.C. GranuFoam silver™, KCI medical) as primary dressing, while the contralateral limb was assigned to a silver impregnated calcium alginate dressings (5 cm x 5 cm Suprasorb A+ Ag™, Lohmann Rauscher) as a reference treatment (REF).

Before application of the treatments, all measurements were performed as described under the subsequent headings. For the REF limb, the silver Ca-alginate dressing was moistened with 10 mL of sterile saline solution (0.9% NaCl). The moistened Ca-alginate dressing was then moulded to the wound to minimize overlap with the surrounding skin. The primary dressing was covered with an elastic retention dressing (glatt Lux™, Mai med) and a standard limb bandage was applied as described earlier. For the NPWT limb, the skin surrounding the wounds was degreased with ether, and adhesive spray was applied. Next, the entire dorsomedial aspect of the metacarpus (including the wounds) was covered with an occlusive polyurethane foil (V.A.C. drape™, KCI medical) and sterile scissors were used to excise the foil at the level of the wounds to free them. A silver impregnated polyurethane dressing (V.A.C. GranuFoam silver™, KCI medical) was cut into a spectacles-like shape to fit both wounds with a bridge in between them, and was applied to the wounds (Fig. 3). The dressing was fixed onto the wounds and sealed airtight with the occlusive polyurethane foil (V.A.C. drape™, KCI medical). A 2 cm opening was cut into the occlusive foil over the bridge halfway the two wounds, and a suction pad was applied (Fig. 3). The suction pad was connected to the canister of the NPWT system (V.A.C. ATS™, KCI medical) and the negative pressure was set at 125 mm Hg in continuous mode. A standard limb bandage was applied over the NPWT bandage, as described for the REF limb. During the NPWT treatment the horses were tethered at both sides of their head to minimize their range of movement and prevent them from lying down. The NPWT system was fixed to the wall at the side of the NPWT-treated limb. The horses had free access to haylage, hay and water during this period and received acepromazine maleate PO (Placivet, Kela, 2ml) four times a day as a mild sedative. The NPWT system and the animals were monitored (e.g. pressure level, leaks and blockades of the system and pain, distress in the animals) every hour during the day and every two hours at night.

Figure 3. A picture of the negative pressure wound therapy bandage.
A silver impregnated polyurethane dressing was cut into a spectacle-like shape to fit both wounds with a bridge in between them, and was applied to the wounds. The dressing was fixed onto the wounds and sealed airtight with the occlusive polyurethane foil and a 2 cm opening was cut into the occlusive foil over the bridge just halfway the two wounds over which a suction pad was applied.

The NPWT and REF treatments were applied for 6 days with a bandage changes at 3 days. Afterwards, both limbs were treated with hydrophilic polyurethane foam dressings (Kendall) with bandage changes every 3 to 7 days. The wounds were monitored for a period of 71 days. The proximal wounds on both limbs were used for the non-invasive measurements, while the distal wounds were biopsied at regular time points to follow up the quality of healing and the inflammatory response. If the granulation tissue exceeded the surrounding skin by 2 to 3 mm, the excess tissue was trimmed with a scalpel blade during a bandage change, after having obtained all necessary samples. For this procedure horses were sedated with detomidine hydrochloride (Detogesic, Zoetis, 10µg/kg) and butorphanol tartrate IV (Torbugesic, Zoetis, 10µg/kg).

Non-invasive measurements

During the bandage changes, on days 0, 3, 6, 9, 14, 19, 24, 29, 36, 43, 50, 57, 64 and 71, the wounds were first clinically assessed using a checklist (table 1). Next, the wounds were cleaned using sterile gauzes moistened with sterile saline solution (0.9 % NaCl), and swabs for quantitative bacteriology were taken using the Levine technique (Levine et al., 1976). Briefly, a rayon tipped swab was rotated 360° between thumb and index fingers centrally over 1 cm^2 of the wound during 5 sec. The swab was placed in a container without medium and labelled with the horse's identification, the date, number of days post-inoculation, and wound reference. The swabs were stored at 4°C for a maximum of 4 hours before they were processed in the laboratory. To evaluate swelling, the circumference of the limb was

measured immediately distal to the proximal wound using a measuring tape. Blood flow was indirectly measured using a thermographic camera (ThermaCam E2, FLIR Systems) as described by Celeste et al. (2011) (chapter 6.1). Wound dimensions (surface area, maximum depth and volume) were measured using a laser beam camera (SilhouetteStarTM, ARANZ Medical Ltd) as described previously (Van Hecke et al., 2015).

Table 1. The checklist used to evaluate the wounds for signs of infection.

If the sign listed was present in the wound, the box next to the description was checked off. The rows with three check boxes distinguish between the different signs listed, while in the rows with one checkbox, a check was placed if either one of the described signs was present.

Exudate (oozed through the third layer of the bandage) clear / sanguineous / purulent			
Red granulation tissue (beefy aspect)			
Yellow necrosis/slough/discoloration of the granulation tissue			
Black necrosis/slough/discoloration of the granulation tissue			
Exuberant granulation tissue			
Oedematous granulation tissue (glassy, shiny aspect)			
Friable granulation tissue (easily bleeding when probing the surface and base of the wound)			
Bone visible or felt with a probe			
Oedema around the wound/ part of the limb /entire limb			
Unpleasant odour			
Pain (it is difficult to touch the wound even when the horse is sedated)			

Invasive measurements

On days 3, 6, 9, 19 and 29 an 8 mm punch biopsy was taken along the wound edge of the distal wounds of both limbs. Horses were sedated with detomidine hydrochloride (Detogesic, Zoetis, 10 µg/kg) and butorphanol tartrate IV (Torbugesic, Zoetis, 10 µg/kg) and about 2mm of skin and/or epithelial border and 6 mm of granulation tissue were included in the biopsy. The location of the biopsy was alternated following a star shaped pattern (Fig. 4), of which the first biopsy site was randomly assigned. The biopsies were placed in a sterile container and labelled with the horse's identification, number of days post-inoculation and wound reference. The biopsies were stored at 4°C for a maximum of 4 hours before they were processed in the laboratory. Half of the biopsy was used for histology to follow up the

inflammatory response and the other half was used for growth factor analysis of transforming growth factor β1 (TGF-β1) and transforming growth factor β3 (TGF-β3).

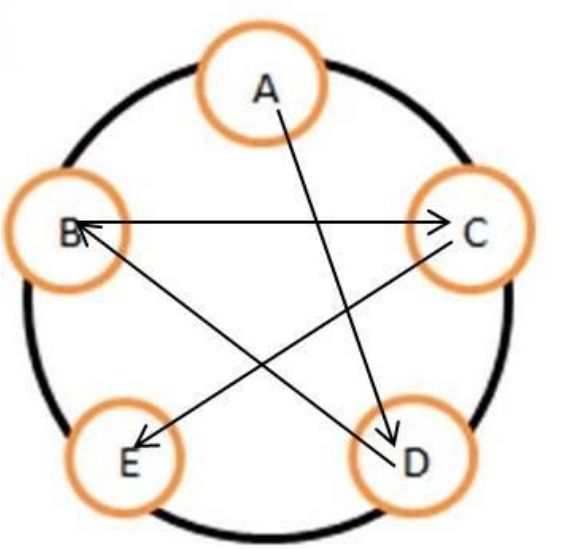

Figure 4. Schematic overview of biopsy scheme.

On days 3, 6, 9, 19 and 29, an 8 mm punch biopsy was taken along the wound edge of the distal wounds of both limbs. About 2 mm of skin and/or epithelial border and 6 mm of granulation tissue were included in the biopsy. The location of the biopsy was alternated following a star shaped pattern, of which the first biopsy site was randomly assigned using a random number generator.

Quantitative bacteriology

The swab tip was cut off with sterile scissors and put into a 1.5 mL Eppendorf tube filled with 1mL of sterile phosphate buffered saline (PBS). The tip was vortexed during 30 seconds and then serially diluted 10-fold in PBS. Five 10µL aliquots of each dilution were spot-plated onto a Columbia agar supplemented with 5 % sheep blood (Oxoid). The Columbia agar plates were incubated in a 5 % CO2 enriched atmosphere at 37°C for 24 hours, after which the colony-forming units (CFU) were counted. The number of CFU per swab was calculated taking the dilution into account.

Growth factor analysis

The tissue samples were aseptically weighed and processed using a standard mammalian cell lysis kit (Sigma Aldrich) containing a protease inhibitor cocktail. Briefly, the biopsy was washed twice in chilled (4°C) sterile PBS and finely diced before adding it to the cell lysis buffer (1mL for 5-20 mg of tissue). The biopsy was incubated in the buffer for 30 minutes on an orbital shaker in a chilled styrofoam box. Next, the biopsy was homogenized using a polytron homogeniser (PT1200 E, Kinematica) and centrifuged in a chilled centrifuge (4 °C, Heraeus multifuge 1SR, Thermo scientific) for 10 minutes at 5000 g. The supernatant was aliquoted and frozen at -20 °C.

For the TGF-β1 and TGF-β3 analysis an equine enzyme-linked immunosorbent assay (ELISA) was used according to the manufacturers' guidelines (SEA124Eq, Cloud Clone corp., for TGF-β1 and SEB949Eq, Cloud-Clone corp., for TGF-β3). All samples were thawed before

processing. The sensitivity of the TGF-β1 and TGF-β3 assay was 28 pg/mL and 6.3 pg/mL respectively.

Histology

Tissue samples were fixed in 4% formaldehyde, embedded in paraffin and sectioned in 4 µm slices. On each sample a Hematoxylin and eosin (HE) and Masson trichrome stain was performed. Microscopic evaluation of all samples was performed by the first author, who was blinded from treatment. The samples were scored based on the scoring system described by Demaria et al. (2011) (chapter 6.1). Briefly, neutrophilic infiltration, tissue oedema, extent of haemorrhage and tissue necrosis were scored from 0-3. The scores of these 4 parameters were added to obtain a histologic acute inflammation score (HAIS; range 0-12). To assess the quality of the granulation tissue the fibroblast proliferation, collagen density and collagen orientation were scored from 0-3. The scores of these parameters were added to a histologic repair score (HRS; range 0-9).

Additionally, the samples were stained using immunohistochemistry for the demonstration of B-cells (polyclonal rabbit anti-CD20, Thermo Scientific, 1:100), T-cells (polyclonal rabbit anti-human CD3, Dako, 1:100), macrophages (monoclonal mouse antibody MAC387, Abcam, 1:100), endothelium (rabbit anti-human von Willebrand Factor, Dako, 1:3200) and myofibroblasts (monoclonal mouse anti-human Smooth muscle actin clone 1A4, Dako, 1:200). Immunolabeling was achieved using a highly sensitive horseradish peroxidase mouse or rabbit diaminobenzidine kit (Envision DAB+ kit, Dako) in an auto-immunostainer (Dako). This kit also blocked endogenous peroxidase. Positive staining was confirmed on light microscopy, and the area percentages (ratio positive stained cells on entire high power field) of three to five (depending on the size of the biopsy) photographed areas (2 or 1 in the superficial layer of biopsy, 1 in the middle layer and 2 or 3 in the deep layer) were calculated per section using LAS V4.0 software (LEICA Microsystems).

Statistical analysis

Statistical analysis was performed using SPSS statistics 20 (IBM). The clinical assessment parameters were analysed over the entire follow up period using a generalized linear model with a binomial probability distribution and a logit link function. The model was corrected for dependent observations by defining horse as a subject effect and leg and time as within subject effects. Clinical parameters were used as dependent variables and respective treatments as independent variables. For the other non-invasive measurements, the follow-up period was divided into 3 time periods, because there were too little observations to analyse each sample point separately: the treatment period from day 0 until 6, the early

period after treatment from day 9 until 29 and late period after treatment including day 36 until 71. An analysis of variance for repeated measures was performed with time period and treatment and their interactions as crossed fixed factors. For the analysis of the histological data, a generalized linear model with multinomial probability distribution and cumulative logit link function was used. The model was corrected for dependent observations by defining horse as a subject effect and leg and time as within subject effects. The scores for the different parameters were used as dependent variable and treatment as independent variable. The area percentages of the immunohistological data were analysed using a generalized linear model with a binomial distribution and a logit link function. Again, the model was corrected for dependent observations by including horse as subject effect and leg and time as within subject effects. The area percentages were used as dependent variable and the treatment as independent variable. The histological and immunohistological data were analysed for all the time points combined (days 3, 6, 9, 19 and 29) to estimate the overall effect. For the growth factor analysis, an analysis of variance for repeated measures was performed with time and treatment and their interactions as crossed fixed factors. A P-value of < 0.05 was considered to be statistically significant. All model assumptions were checked and met.

Results

Wound dimensions

There was no significant difference in the wound surface area between the two treatments. However, for both treatments the wound area was significantly larger during the treatment period compared to the early and late period after treatment, and in the early period compared to the late period ($P < 0.05$) (Fig. 5).

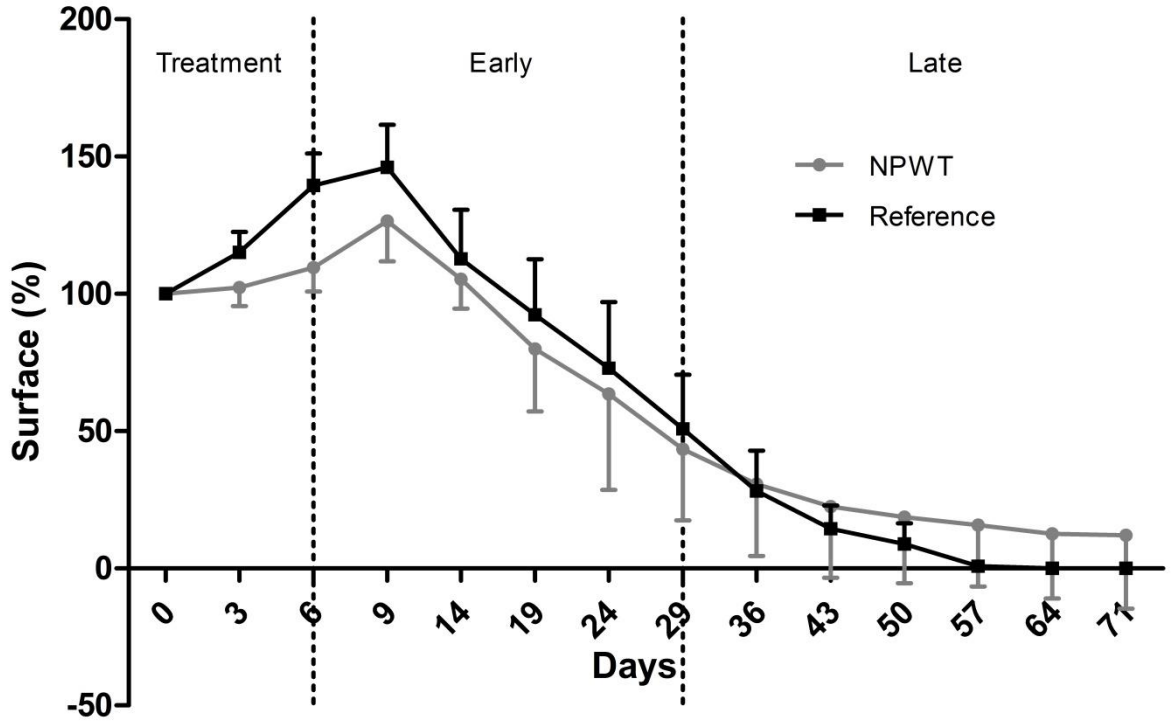

Figure 5. Evolution of wound surface area over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 6 (Treatment), the early period after treatment from day 9 until 29 (Early) and late period after treatment including day 36 until 71 (Late). NPWT: negative pressure wound therapy. Reference: silver Ca-alginate dressings.

For wound volume, there was no significant difference between treatments, but within treatments a significant effect of time was seen (P = 0.032) (Fig. 6). However, when comparing the different time periods to each other, the decrease from the treatment period to the early period after was no longer significant because of the Bonferroni correction for multiple comparisons.

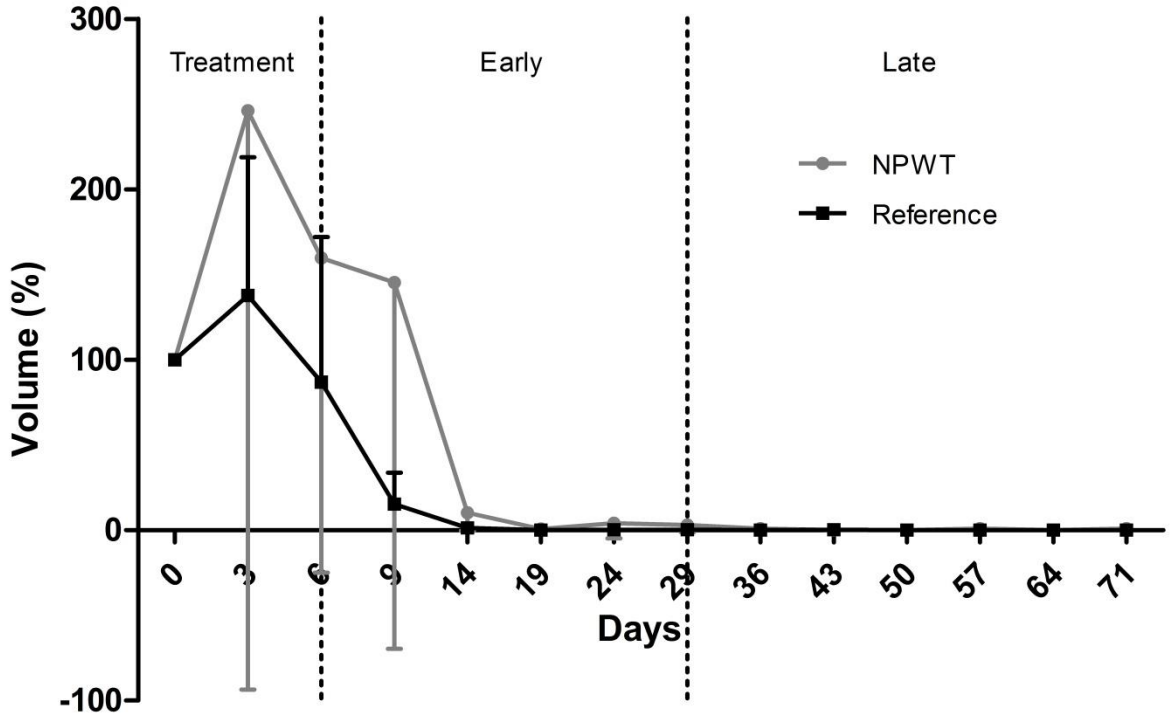

Figure 6. Evolution of wound volume over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 6 (Treatment), the early period after treatment from day 9 until 29 (Early) and late period after treatment including day 36 until 71 (Late). NPWT: negative pressure wound therapy. Reference: silver Ca-alginate dressings.

No significant difference in maximum depth was seen between the REF and NPWT treatment. For both treatments, the maximal wound depth was significantly larger during the treatment period compared to the early period after treatment (P = 0.003) (Fig. 7).

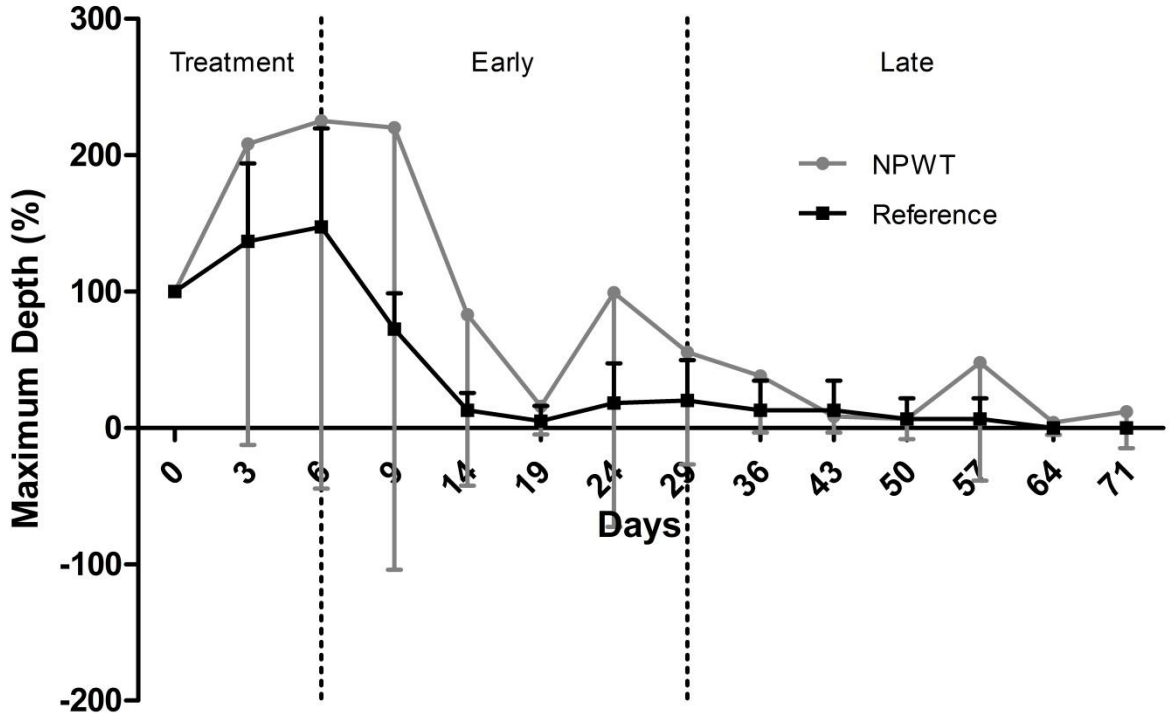

Figure 7. Evolution of wound maximal depth over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 6 (Treatment), the early period after treatment from day 9 until 29 (Early) and late period after treatment including day 36 until 71 (Late). NPWT: negative pressure wound therapy. Reference: silver Ca-alginate dressings.

Clinical assessment

The analysis of the clinical assessment showed a significant higher chance for sanguineous exudate and red granulation tissue in NPWT wounds ($P < 0.001$ and $P = 0.002$ respectively). On the other hand, REF wounds were significantly more likely to display purulent exudate and oedematous granulation tissue ($P = 0.019$ and $P < 0.001$ respectively). Exuberant granulation tissue was trimmed 5 times in REF wounds and 8 times in NPWT wounds. Exuberant granulation tissue was mostly present in the early and late period after treatment.

Histology

There was no significant difference in the histological acute inflammation score or its different parameters (tissue oedema, neutrophilic infiltration, extent of haemorrhage and tissue necrosis) between the REF and NPWT treatment. There was also no significant difference in HRS, nor in its different parameters (fibroblast proliferation, collagen density and orientation) between the two treatments. Additionally, the presence of B-cells, T-cells, macrophages,

myofibroblasts and neovascularisation was not significantly different between the NPWT and REF wounds.

Bacterial load, limb swelling and wound temperature

There was no significant difference in bacterial load between treatments. For REF wounds, the bacterial load significantly decreased from the treatment period to early period after treatment (mean decrease of 41% $P < 0.001$), followed by a significant increase from the early period to the late period after treatment (mean increase of 18% $P = 0.004$). However, compared to the treatment period the bacterial load was still significantly lower in the late period after treatment (mean difference of 22 % $P < 0.001$) (Fig. 9). For NPWT wounds, the bacterial load was significantly higher in the treatment period than in the early and late period after treatment (mean difference of 42% and 43% respectively both with $P < 0.001$). There was no significant difference in bacterial load between the early and late period after treatment (Fig. 8).

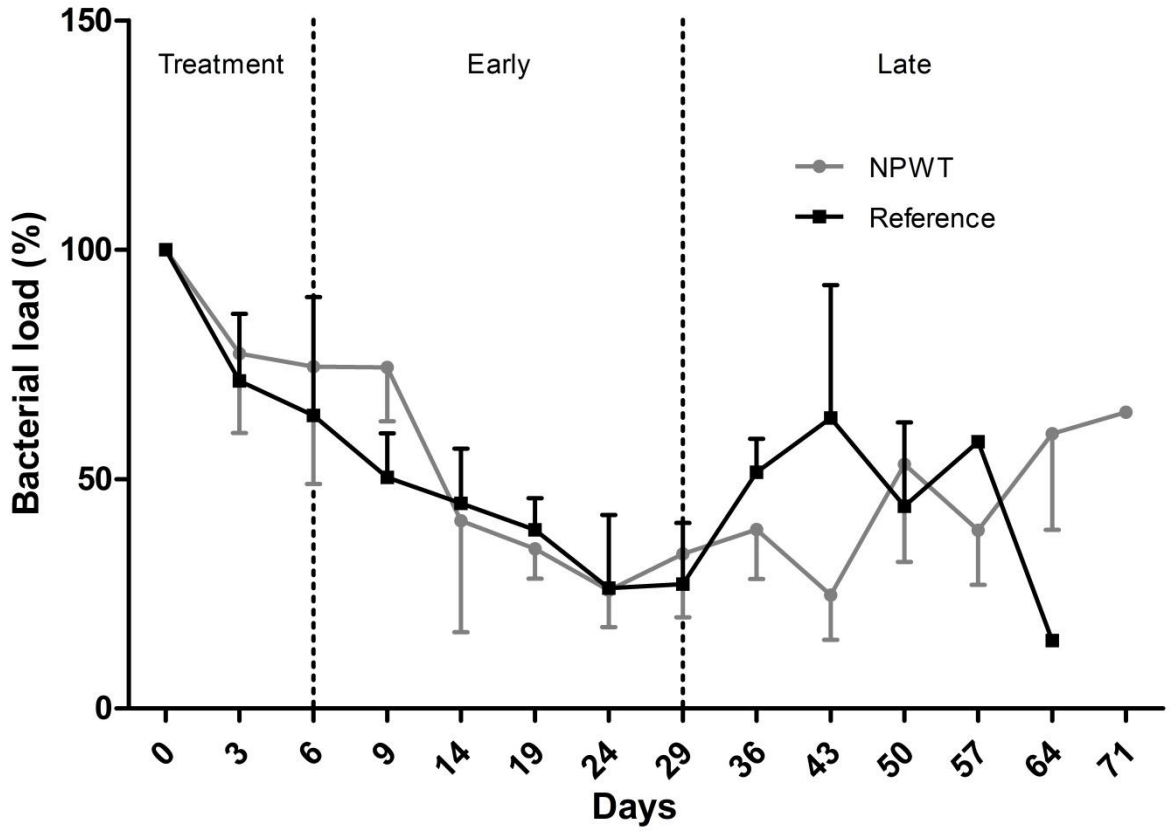

Figure 8: Evolution of bacterial load over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 6 (Treatment), the early period after treatment from day 9 until 29 (Early) and late period after treatment including day 36 until 71 (Late). NPWT: negative pressure wound therapy. Reference: silver Ca-alginate dressings.

Swelling of the limbs was not significantly different between the REF and NPWT treatment. For both treatments, the limbs were significantly more swollen during the treatment period compared to the early period after treatment (P = 0.023).

There was no significant difference in temperature, thus indirectly in blood flow, between treatments. However, for both treatments, the temperature was higher during the treatment period compared to the late period after treatment (mean difference of 24 % P= 0.039) (Fig. 9).

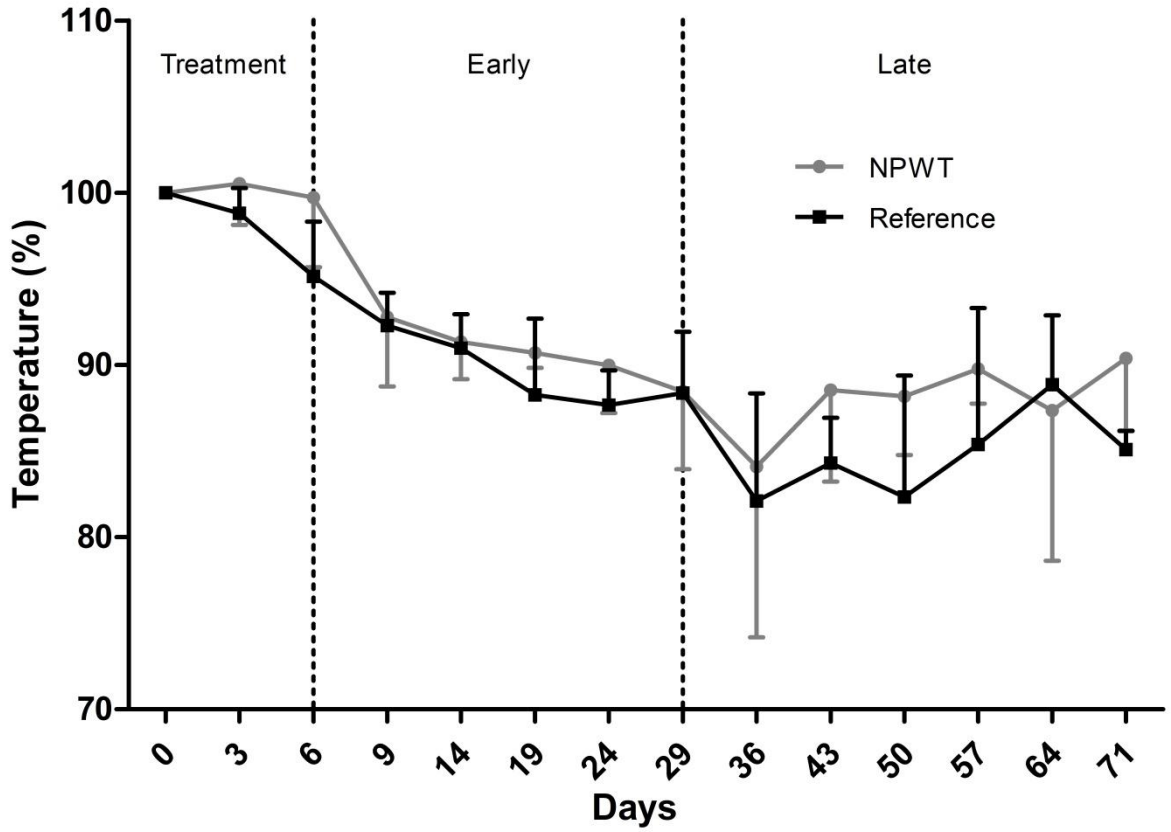

Figure 9: Evolution of wound temperature (an indirect measure for blood flow) over time compared to day 0 in percentages (mean + or – SD) for both treatments.

The follow-up period was divided into 3 time periods: the treatment period from day 0 until 6 (Treatment), the early period after treatment from day 9 until 29 (Early) and late period after treatment including day 36 until 71 (Late). NPWT: negative pressure wound therapy. Reference: silver Ca-alginate dressings.

Growth factor analysis

There was no significant difference in TGF-β1 and TGF- β3 concentration between NPWT and REF wounds. For TGF-β1, there was also no significant difference in concentration within treatment groups between time points. For TGF-β3, the concentration on day 29 was significantly lower compared to day 9 (P = 0.017) and day 19 (P = 0.032) for the REF wounds.

Discussion

The aim of the study was to investigate the clinical relevance of NPWT for the treatment of contaminated distal limb wounds in horses. Therefore, we modified the wound model used in chapter 6.1. This wound model has a larger surface area than most models of other equine studies on second intention wound healing (± 10 cm^2 vs 1- 7cm^2) (Wilmink et al., 1999; Theoret et al., 2001; Theoret et al., 2002; Monteiro et al., 2009; Bischofberger et al., 2011; Broeckx et al., 2015; Bischofberger et al., 2016), thus improving the similarity with real life traumatic wounds. However, traumatic wounds are always contaminated with micro-organisms, while most studies on equine wound healing use an aseptic model (Wilmink et al., 1999; Theoret et al., 2001; Theoret et al., 2002; Monteiro et al., 2009; Provost, 2012; Broeckx et al., 2015). To the authors' knowledge, only one research group worked with a contaminated equine wound model to evaluate wound treatments (Bischofberger et al., 2011; Bischofberger et al., 2013; Bischofberger et al., 2016). This research group used fresh faeces, applied for 24 hours, to inoculate the wounds. The number and different species of bacteria in faeces varies between horses and even in one horse over time, making it hard to create a standardized inoculated wound model using faeces. This variation reduces repeatability and comparability between studies. Moreover, only the latest study of this research group used quantitative bacteriology to follow up the bacterial load in their model, thus the effect of the inoculation procedure in their first two studies is uncertain (Bischofberger et al., 2011; Bischofberger et al., 2013; Bischofberger et al., 2016). In our study however, we wanted to use a standardized inoculated wound model. To standardize the inoculation, two common equine wound pathogens were chosen (a gram positive and a gram negative species) to represent a normal wound flora. Quantitative swabs were taken during the inoculation procedure to ensure effectiveness (data not shown here). The day before commencement of the treatments, a mean of 6.5 x 10^5 CFU per swab were present in the wounds indicating an efficient inoculation. Our inoculated wound model was well tolerated by the horses. They did not show any signs of discomfort or pain in the stable and were not lame. During the first bandage changes (day 0-6) some horses reacted during wound cleansing, but this was resolved by sedating the horses. Therefore, the authors

considered this inoculated wound model an efficient, safe and feasible alternative over most common wound models in other equine studies. To improve this wound model, more bacterial species could be used. Moreover, the subcutis could be partially heated with an electric scalpel to cause necrosis during surgery or foreign objects could be introduced in the wounds, such as sterile sand, after the inoculation process. These measures would further enhance the similarity with real life traumatic wounds.

The bacterial load of the wounds before commencement of the treatments, as confirmed with quantitative swabs (mean of 6.5 x 10^5 CFU per swab), indicated not only an efficient contamination, but even an infection of the wounds. After all, in chapter 4, we found that a value of 1.2 x 10^5 CFU per swab corresponds with the critical value of > 1 x 10^5 CFU per gram of tissue for the quantitative biopsy. Moreover, on the clinical assessment before commencement of the treatments (day 0), all but one wound displayed purulent exudate and yellow discoloured granulation tissue. Most wounds also displayed red granulation tissue and in about half of the wounds the granulation tissue was friable. These clinical signs indicated an inflammation of the wounds. Associated with the heavy bacterial load as found with the quantitative swabs, the inflammation was probably due to infection.

TGF-β3 concentrations were not different between treatments, but over time the concentrations displayed a significant dip at day 29 compared to day 9 and 19. Theoret et al. (2002) reported in their study on the evolution of TGF-β concentrations in normally healing wounds and wounds with exuberant granulation tissue that TGF-β3 concentrations displayed a slow increase over time with a peak value on 10 days. Therefore, the results of this study are quite similar to the results of Theoret et al. (2002). The peak of TGF-β3 concentrations around day 9-10 confirms the role of this growth factor in the later stages of wound healing, where it is reported to be important in limiting the fibrotic process (Shah et al., 1995).

The only significant differences between treatments were seen for the clinical parameters. NPWT wounds displayed more red granulation tissue and sanguineous exudate compared to REF wounds. This concurs with the known effects of NPWT in men, where the therapy stimulates blood flow and angiogenesis, which could explain the reddish appearance of the granulation tissue and the presence of blood in the exudate (Mouës et al., 2011). However, in our study, no difference between treatments was seen for wound temperature as measured with thermography or angiogenesis as evaluated on the von Willebrand factor stain. In contrast, REF wounds showed more oedematous granulation tissue and purulent exudate. Again, this difference was not confirmed on histology by a higher degree of infiltration of neutrophils or oedema. Since the histological and immunohistochemical stains were performed at only five time points, whereas the clinical assessment and thermographic

images were performed on 14 time points, it is possible that the differences on histology were not profound enough to be significant. This could possibly be resolved by including more horses in the study. However, only a limited number of horses was used in this study because of practical and ethical considerations. In our previous study on NPWT using an acute non-inoculated wound model, this number of animals was sufficient to detect significant differences.

The bacterial load in this study did not differ significantly between the two treatments groups. However, there was a significant decrease in bacterial load from the treatment period to the early period after treatment for both groups. Because of the large amount of sample days and the limited number of animals it was not possible to analyse the bacterial load for each sample point. However, on figure 8 a steep reduction of bacterial load is seen for both treatments from day 0 to 3, indicating an antibacterial effect of both silver dressings. Since there was no significant difference between the treatments, the decrease of bacterial load in this period is probably due to merely the antibacterial activity of the silver ions in the dressings and not the suction effect of the NPWT system. In the early period after treatment, exuberant granulation tissue was present in some horses, which had to be excised to the level of the surrounding skin for the wound healing to proceed. This debrides the wound and could be responsible for the drop in bacterial load in this period. For REF wounds, an increase of bacterial load was seen in the late period after treatment compared to the early period after treatment. However, in the late period after treatment there was a lot of variation in the bacterial load, thus the observed increase could be potentially coincidental or the result of the increased difficulty to swab the wound as the wound surface area decreases.

During this study, only quantitative bacteriology was performed. This provided a good overview of the evolution of the bacteria load over time for the two treatments, but it did not determine the evolution for the two bacterial species separately. Therefore, qualitative bacteriology would have been a valuable addition to this study. It would have been interesting to see if one of the species (*P. aeruginosa* or *S. aureus*) would have taken to upper hand or even disappears out of the wounds over time and if this differed between the two treatments. These results could than have been compared with the study on human clinical wounds of Mouës et al. (2004). They reported that the overall bacterial load was stable during NPWT, but that there was a decrease of nonfermentative gram negative rods and an increase of *Staphylococcus aureus*. The bacterial load in our study was not stable, but additionally it could have been possible there was also a shift in bacterial species.

In contrast to our study using the acute non-inoculated wound model (chapter 6.1), no significant differences were seen between NPWT and REF wounds for any of the other

investigated parameters. In the study on acute wounds, NPWT-treated wounds retracted less during the early stages of healing. This was not seen in this study on inoculated wounds. A possible explanation could be that the inoculated wounds were older when the treatment commenced compared to the acute wounds from our previous study (4 days vs 1 day) (chapter 6.1), causing the wound to already be partially retracted. Moreover, the presence of bacteria also stimulates retraction of a wound (Bischofberger et al., 2013). There was also no difference in maximum depth between treatments, whereas in the study on acute wounds, NPWT-treated wounds were significantly deeper in the treatment period and early period after treatment. As mentioned earlier, the inoculated wounds in this study were older and thus already partially filled with granulation tissue at the commencement of the treatments. Additionally, the presence of bacteria could enhance the inflammatory response, making the wounds to fill up faster with granulation tissue and eliminate any treatment effect as seen in the acute wounds. However, there were some differences in study design between the two studies which could also explain the differences in results. The treatment period in this study only consisted out of 6 days compared to 9 days in our study on acute wounds. The treatment period was shorter in this study because we used the alginate and NPWT treatment until the wound was filled with granulation tissue, as we would do in clinical practice. Since the inoculated wounds were older to begin with, they were filled faster with granulation tissue compared to the acute wounds. Additionally, different wound dressings were used in this study. Both dressings used in the treatment period were impregnated with silver ions. The silver in the polyurethane foam was present as uniformly distributed micro-bonded metallic silver, while the silver in the alginate dressing was present as a silver alginate fibre woven between the Ca-alginate fibres. Both dressings release ionic silver after coming in contact with wound exudate. These silver ions could have potentially influenced the wound healing process. However, since both dressings contained the silver ions, it was expected that the differences seen in the study on acute wounds between the two treatments would remain unaltered.

Conclusion

No real advantage could be detected for treating contaminated distal limb wounds in horses with NPWT using a silver impregnated polyurethane foam over silver calcium alginate dressings. NPWT wounds displayed a significant higher chance for sanguineous exudate and red granulation tissue, whereas REF wounds were significantly more likely to display purulent exudate and oedematous granulation tissue. However, no significant difference between the two treatments could be detected for any of the other investigated non-invasive parameters. Additionally, there was also no significant difference in histological inflammation

and repair scores, nor in the presence of B-cells, T-cells, macrophages, myofibroblasts, neovascularisation and growth factors.

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GENERAL DISCUSSION

Traumatic wounds in horses are a common concern for both equine practitioners and horse owners. Although the best aesthetic and functional results of wound healing are acquired with primary closure, traumatic wounds in horses often dehisce or are left open to heal by second intention because of massive tissue loss, pronounced bacterial contamination or a high skin tension. Moreover, if the wound is located at the distal limb (below hock or carpus) the wound healing is often complicated, leading to an extended healing time with an increased morbidity for the horse and costs for the owner. The inflammatory response in these distal limb wounds is inefficient, which causes a chronic inflammation and an improficient wound debridement (Wilmink, 2008). Therefore, the chance of wound infection in equine distal limb wounds is increased (Wilmink, 2008). Wound healing is even further impaired because of a decreased oxygenation and more occlusion of microvessels, which contribute to the chronic inflammation and exuberant granulation tissue formation (Lepault et al., 2005; Celeste et al., 2011). Negative pressure wound therapy (NPWT), which is frequently used in human medicine to treat chronic wounds (Vig et al., 2011), could potentially address certain of these issues. Indeed, in human medicine, NPWT has been shown to increase local blood flow, stimulate angiogenesis, granulation tissue formation and cell proliferation. It also reduces size and complexity of a wound and there are indications that NPWT modulates the inflammatory response (Mouës et al., 2011). However, no controlled studies in horses are available which assess the effect of this treatment on distal limb wounds. Therefore, the general aim of this PhD study was to assess the influence of NPWT on second intention healing in equine distal limb wounds. However, before commencing with controlled *in vivo* studies, the methods necessary to follow up wound healing and the best choice of wound dressing had to be further refined. In the following chapter the main accomplishments of this PhD research will be discussed in a broader context with additional focus on the possibilities for future research.

Problems of equine second intention wound healing and the latest developments of the solutions

In the general introduction of this PhD study (chapter 1), the problems of equine second intention wound healing were discussed. The main issue of equine second intention wound healing is the weak but protracted inflammatory response, which leads to a series of well - known complications such as chronic inflammation, wound infection, exuberant granulation tissue formation, increased wound retraction, etc.… (Wilmink, 2008). Therefore, there is a continuous search for treatments for equine distal limbs wounds to address these complications. As mentioned in chapter 1, some studies use new techniques such as silver (Kelleher et al., 2015) or biological therapies (Monteiro et al., 2009, Broeckx et al., 2015), other use old techniques such as honey (Bischofberger et al., 2013) or negative pressure (Jordana et al., 2011). However, most of these wound treatments are first tested in one animal, of which the outcome is then published as case reports or low evidence controlled studies (Carter et al., 2003; Gemeinhardt and Molnar, 2005; Jordana et al., 2011; Iacone et al., 2012; Spaas et al., 2013; Broeckx et al., 2014). Another common publication type is case series (Rijkenhuizen et al., 2005; Morrison, 2007; Sherman et al., 2007; Lepage et al., 2012). The latest wound treatments are thus often used based on empirical findings and not on evidence based medicine. Well-designed controlled studies are needed to further evaluate these new wound treatments. These studies have to include sufficient number of animals, so relevant conclusions can be made. Additionally, care has to be taken to sufficiently describe the used methods and standardise them, so experiments can be repeated and comparison between different studies is possible and relevant. A well-thought-out reference treatment is also a very important. In a preliminary study, untreated wounds can be used as a control/reference (Bischofberger et al., 2011), but often this leads to optimistic results since most treatments will better than no treatment at all. Therefore, new treatments should also be tested compared to a relevant reference treatment.

For NPWT specifically, the research in horses mainly entails case reports (Gemeinhardt and Molnar, 2005; Rijkenhuizen et al., 2005; Jordana et al., 2011), but based on human medicine, NPWT shows potential to stimulate wound healing in equine distal limb wounds (Birke-Sorensen et al., 2011; Krug et al, 2011; Vig et al., 2011; Mouës et al., 2011). Controlled studies on NPWT in horses are however lacking. Therefore, two controlled experimental studies were performed in this PhD study to address this gap in the literature (chapter 6.1 and chapter 6.2). Before these studies could be started, some methods necessary to perform these studies had to be refined (chapters 3, 4 and 5).

Refinement of the used methods

Wound measurement in horses

Measuring wound dimensions is an essential part to monitor wound healing (Little et al., 2009). In veterinary clinical practice, wound dimensions are often only evaluated visually without making any records, so the oversight on the healing process is easily lost. Moreover, estimations of wound dimensions are highly subjective and thus not desirable in clinical practice and certainly not in research.

In human medicine, several techniques for objective wound measurement have been investigated. These can be categorized as either 2D techniques which measure length, width, area and/or wound circumference, or 3D techniques which also measure wound depth and volume in addition to the standard 2D measurements. The most common 2D techniques are the ruler technique (Langemo et al., 1998; Shaw et al., 2007; Little et al., 2009; Shetty et al., 2007; Foltynski et al., 2013), wound tracing on a transparent sheet with manual counting of square centimetres (Langemo et al., 1998; Little et al., 2009), digitalized counting (Shaw et al., 2007; Little et al., 2009; Foltynski et al., 2013; Foltynski et al., 2014) and photoplanimetry (Shaw et al., 2007, Shetty et al., 2007; Bhedi et al., 2013). The most common 3D techniques are the use of the Kundin formula (Langemo et al., 2001; Little et al., 2009), laser beam based techniques (Little et al., 2009; Miller et al., 2012) and stereophotogrammetry (the use of 3 pictures or more from different angels to construct a 3D image of the wound) (Langemo et al., 2001; Little et al., 2009; Labens and Blikslager, 2013). Presently, the laser beam based techniques and stereophotogrammetry are considered the most precise and accurate techniques but also the most expensive (Langemo et al., 2001; Little et al., 2009).

In equine medicine, digital photoplanimetry (DP) is the most frequently used technique to evaluate wound dimensions (Monteiro et al., 2009; Tóth et al., 2011; Bischofberger et al., 2013, Tracey et al., 2014; Kelleher et al., 2015). However, this technique cannot measure either the depth or volume of a wound. Moreover, before this PhD study, the accuracy or precision of this technique was unknown, reflecting a gap in the research on wound measurement techniques in veterinary medicine. To fill this gap in the literature and to evaluate one of the more accurate human techniques in horses, we performed a study to compare DP to a laser beam (LB) camera (SilhouetteStar, Aranz Medical ltd.). However, DP is a 2D measurement technique, so we supplemented it with a manual measurement of the maximum wound depth, thus allowing the wound volume to be calculated using the validated Kundin formula and making this DP-based method (DPB) more equal to the laser beam camera (Kundin, 1989; Langemo et al., 1998, Langemo et al., 2001).

Only 3D wound measurement techniques were evaluated in this PhD study, since granulation tissue formation plays a crucial role in equine wound healing, especially in second intention healing wounds (Theoret, 2008). In the early stages of healing, granulation tissue starts to form at the base of the wound, but the wound surface area remains unchanged (Little et al., 2009), so depth and volume measurements are essential to monitor progress. Additionally, because stimulation of granulation tissue formation is one of the main effects of NPWT in human medicine (Mouës et al., 2011), a 3D wound measurement technique was the pre-eminent method to follow up wound healing for this PhD study. In fact, all future studies on second intention wound healing in horses would benefit from using a 3D measurement technique, as they provide a more complete and accurate evaluation of wound healing than a 2D wound measurement technique.

The LB camera proved to be the superior technique to measure wound area and circumference in horses. Moreover, this technique was also more accurate in determining maximum wound depth. The LB camera was thus chosen as the *modus operandi* to measure wound dimensions in our *in vivo* studies on NPWT (Chapter 6.1 and 6.2), even though the DPB method had a higher accuracy and precision for the determination of wound volume. However, since the study in chapter 3 was performed on cadavers, we expected a greater variability and less accuracy with the manual depth measurement of the DPB method in live horses. This would subsequently also influence the variability and accuracy of the wound volume calculations for this technique. Moreover, the LB camera was more user friendly, making frequent repeated measurements of wound dimensions more feasible. Nevertheless, to objectively confirm a higher accuracy and precision of the LB camera compared to the DPB this should be tested in live horses, preferably with clinical wounds. Additionally, since the accuracy and precision of the LB camera was not specifically tested on circumferential wounds, this could be investigated in the same study.

Monitoring bacterial load

Besides monitoring the wound dimensions during the healing process, a follow up of bacterial load is also important. Each traumatic wound is contaminated with micro-organisms (Provost, 2012). This means that micro-organisms are present in the wound bed, but are not (yet) replicating. This contamination can proceed into a colonization, in which the micro-organisms start replicating, but do not cause damage to the host. When the wound is critically colonized, the replicating organisms start to cause harm to the host resulting in a local immune response. At this stage the wound healing can start to delay, but the clinical signs are not obvious. However, when this critical colonization proceeds into an infection the damage increases, leading to a local and systemic host immune response and deterioration

of the wound (Bonham, 2009). Infection has several detrimental effects on wound healing such as enlarging of the wound, a decreased efficiency of the microvasculature near the wound edges, an increase of the inflammatory response and a prolongation of this response, the stimulation of production of proteolytic enzymes and the release of endotoxins inhibiting local growth factors and collagen production (Stashak, 2008a). Therefore, recognition of wound infection is an essential skill when evaluating wound healing.

In human medicine, the golden standard to diagnose wound infection in research is quantitative bacteriology on a tissue biopsy of the wound. An excess of 10^5 CFU/g of tissue is associated with a delayed wound healing (Robson, 1997; Serena et al., 2006, Bonham, 2009; Siddique and Bernstein, 2010). In equine medicine, this guideline has been adopted (Adam and Southwood, 2006) and confirmed in a study of Peyton and Connelly (1983). However in equine practice, the diagnosis of wound infection is mainly based on clinical signs (Hendrickson, 2004). Unfortunately, there is no consensus or evidence on which clinical signs to use to diagnose wound infection in horses. The classic signs of infection (erythema, heat, pain, swelling and function loss) are less useful in horses because their skin is hairy and pigmented. Moreover, equine wounds are often left open to heal by second intention with a healing curve which is similar to that of chronic wounds in men, making the classical signs to diagnose infection less relevant (Gardner et al., 2001). Additionally, less invasive alternatives to the quantitative tissue biopsy in horses were not yet investigated before this PhD study.

In chapter 4, we found that quantitative bacteriology performed on a wound swab taken according to the Levine technique is good alternative for a quantitative tissue biopsy to determine bacterial load in an equine second intention healing wound. It was found that a value of > 1.2 x10^5 CFU per swab corresponded with the critical value of > 1 x 10^5 CFU per gram of tissue for the quantitative biopsy. Therefore, this technique was used to follow up the bacterial load in the *in vivo* studies on NPWT in this PhD study (chapters 6.1 and 6.2). Moreover, only black granulation tissue was significantly more prominent in the wounds with a higher bacterial load. Therefore, clinical signs on their own without a concurrent evaluation of the bacterial load were considered not useful to evaluate bacterial bioburden in wounds. The limited relevance of clinical signs to evaluate bacterial bioburden in wounds is a controversial subject in literature (Robson, 1997; Bowler, 2003; Serena et al, 2006). There are two main movements in human and veterinary literature, those who consider clinical signs the standard to diagnose wound infection (Bowler et al., 2001; Bowler, 2003, Westgate et al., 2010; Kallstrom, 2014) and those who consider quantitative bacteriology a valuable asset to diagnose wound infection (Robson, 1997; Robson, 2003; Gardner et al., 2001; Serena et al., 2006). To the authors' opinion quantitative bacteriology is a valuable and more

objective technique to diagnose wound infection, especially in horses. Diagnosis of wound infection based on clinical signs is not based on the mere presence of bacteria but on the immune response of the host. This immune response can be triggered by other factors such as foreign objects, or in the case of horses, the immune response at the distal limb has an innate divergence (Wilmink, 2008). These wounds indeed often show clinical signs of infection without necessarily having a high bacterial load. A clinical assessment of equine wounds is valuable to get a general overview of the wound healing progress, but it is subjective and often subdue to a lot of variation. Especially in research, one has to strive to a more objective evidence based approach of the evaluation of wound parameters. Therefore, we chose to use a combination of quantification of bacterial load and a clinical assessment to evaluate the wound healing in our *in vivo* studies (chapter 6.1 and 6.2).

In equine studies on wound healing, evaluation of bacterial presence is often lacking (Monteiro et al., 2009; Broeckx et al., 2014; Broeckx et al., 2015) or is only performed qualitatively (Bischofberger et al., 2011, Bischofberger et al., 2013; Tracey et al., 2014). Qualitative bacteriology has its merits, especially in equine practice, for the choice of antimicrobials. In research, it can provide useful information on the types of bacteria which spontaneously colonize a wound or the evolution of deliberately applied bacteria. In our *in vivo* studies (chapter 6.1 and 6.2) qualitative bacteriology was not performed from practical considerations and budgetary restrictions. However, it would have been interesting to see which type of bacteria spontaneously colonize the wounds in the acute model (chapter 6.1) and the evolution of the bacteria in the inoculated wound model (chapter 6.2). However, our main goal was to assess the bacterial load as this a more reliable parameter to follow up wound contamination and infection than purely qualitative bacteriology (Robson, 1997). Future research on equine wound healing should always include a quantitative follow up of bacterial load, preferably accompanied with a clinical assessment of the wound and if possible qualitative bacteriology. All the used methods have to be described thoroughly, so studies can be reproduced or comparisons can be made between different studies. For future studies on the follow up of bacterial load, other techniques such as enzyme analysis would also be explored (Blokhuis-Arkes et al., 2015). Additionally, our present study (chapter 4) can be expanded by investigating more wounds with a higher bacterial load, or using an inoculated wound model with different levels of contamination to investigate the validity of certain clinical signs, quantitative and semi-quantitative swab techniques or their combination. It would also be interesting to investigate the inter-and intra-reliability of these techniques. However, these future studies should always include a golden standard such as the quantitative tissue biopsy as a reference. Another reference option is monitoring the progression of the wound healing through the follow-up of wound dimensions.

Choice of the primary wound dressing

In chapter 5 the antibacterial effect of the three most commonly used primary dressings with NPWT was tested. This study demonstrated that the polyvinyl alcohol foam combined with NPWT had the best antibacterial effect *ex vivo* against both *Pseudomonas aeruginosa* and methicillin resistant *Staphylococcus aureus* (MRSA). The silver impregnated polyurethane foam with NPWT had the second best anti-bacterial activity against *P. auruginosa,* but did not differ in antibacterial activity against MRSA from the reference treatment and NPWT using a standard polyurethane foam. A study on goats (Lallis et al., 2010), saw similar results in a contaminated open fracture model. They reported that NPWT using a gauze or foam as primary dressing reduced *Pseudomonas aeruginosa* compared to the wet-to-dry control/reference dressing over a 6 day period. However, there was no difference in bacterial load between the NPWT groups and the control/reference group for *Staphylococcus aureus.* Unfortunately, the type of foam or gauze dressing used with NPWT was not mentioned in this study. Lallis et al. (2010) speculated that *S. aureus* did not decrease because this bacterial species is more virulent than *P. aeruginosa*, which is more an opportunistic pathogen. They also mentioned that the decrease in bacterial load was probably attributed to the increase in blood flow and granulation tissue formation as a result of NPWT. However, these two parameters were not measured in the study of Lallis et al. (2010), making this statement hypothetical.

Based on the results of our *ex vivo* study (chapter 5), a more solid choice of wound dressing could be made for the *in vivo* NPWT study with our inoculated wound model (chapter 6.2). The most logical choice would have been the polyvinyl alcohol (PVA) foam, as this gave the highest antibacterial effect. However, when using this foam on clinical patients with exudative wounds, we encountered difficulties with the NPWT system blocking several times during the treatment. This is probably due to the smaller pore size of the PVA foam (60-270 µm vs. 400-600 µm in the black standard polyurethane foam). Therefore, we decided to use to the silver impregnated polyurethane foam for our *in vivo* study. This silver foam was also more relevant to compare to the reference calcium alginate dressing, which also contained silver. After the study, the manufacturer mentioned that the blockades were also seen in humans when using the PVA foam. They advised placing a small piece of standard black polyurethane foam between the suction pad and the PVA foam to decrease these blockades. Therefore, it still would be interesting to test these PVA foams with NPWT *in vivo* with this altered study design. Moreover, these PVA foams have, to the authors' knowledge, not been tested yet in a controlled *in vivo* study in veterinary or human medicine.

The *ex vivo* wound model in chapter 5 provides an ethically interesting alternative to experimental animals for testing the antibacterial effect of different types of wound dressings. This model can for example be used to test the antibacterial activity of different honey dressings, silver dressings, or to test several types of instillation fluids in the context of NPWT with instillation. Based on this model, a first selection can be made of dressings which look promising to test in an experimental or clinical study. Another advantage if this model is the presence of intact skin around the wounds, which simulates the complex structure of *in vivo* tissue better than monolayer cell lines (Lansdown, 2004). A disadvantage of this model is the rapid onset of decay of the tissues. The skin starts to decay 12 hours after asystole (Kagan et al., 2005). To retard this process, the model can be perfused with a nutrient tissue culture medium instead of a saline solution. However, this will simultaneously stimulate to growth of bacteria. Clearly, this model cannot replicate the complex environment of a real life traumatic wound. There is also no presence of an immune response or progression to wound healing, but it can give an impression of the antibacterial activity of different dressings placed on real tissues. This model can also be adapted by using other bacterial species or a combination of bacteria.

The influence of negative pressure wound therapy on second intention wound healing in equine distal limbs

Acute non-inoculated wound model

In chapter 6.1, we tested the effect of NPWT on second intention healing in an acute non-inoculated wound model of the distal limb. This wound model was larger than the wound models used in most equine studies (± 10 cm² vs 1-7 cm²) (Wilmink et al., 1999a; Wilmink et al., 1999b; Theoret et al., 2001; Theoret et al., 2002; Lepault et al., 2005; Monteiro et al., 2009; Bischofberger et al., 2011; Celeste et al., 2011; Bischofberger et al., 2013; Broeckx et al., 2015; Kelleher et al., 2015; Bischofberger et al., 2016). The main reason for choosing a larger wound model was that this is a more realistic representation of a real life traumatic wound. When the wound size of formerly used wound models is extrapolated from a mean horse of approximately 500 kg to a human with a mean weight of 70 kg, the smallest wound of 1 cm² would only be 0.14 cm² in a human and the largest (7cm²) about 1 cm². Moreover, even our wound model (± 10 cm²) is still relatively small compared to real traumatic wounds in horses. In our wound model, we also chose a circular shape compared to a rectangular or square shape of most wound models (Wilmink et al., 1999a; Wilmink et al., 1999b; Theoret et al., 2001; Theoret et al., 2002; Lepault et al., 2005; Monteiro et al., 2009; Bischofberger et al., 2011; Celeste et al., 2011; Bischofberger et al., 2013; Broeckx et al., 2015; Kelleher et al., 2015; Bischofberger et al., 2016). Again, this is a more realistic representation of a real life

traumatic wound. In human medicine, a deviation of a rectangular shape is also implied with the validated Kundin formula. This formula is used to calculate the wound volume when the surface area and maximum depth are known. It assumes a wound shape somewhere between a sphere and a cylinder (Langemo et al., 2001). We tested this formula in chapter 3 and saw a good accuracy and precision for the measurement of wound volume compared to our reference standard, indicating a good reliance of this formula to irregular wound shapes.

In chapter 6.1, it was shown that our wound model was well tolerated by the horses. They did not show any signs of discomfort or pain in the stables and were not lame. During the first bandage changes (day 0-9) some discomfort was noted during wound cleaning, which was resolved by sedating the horses. This wound model was thus ethically acceptable while being more realistic compared to currently used wound models in horses. The use of acute non-inoculated wounds also permitted the evaluation of the effect of NPWT on second intention wound healing without the added variability of micro-organisms. Additionally in chapter 6.1, we wanted to investigate the effect of NPWT on as much wound healing parameters as possible to increase the value of our study. Most equine studies only investigate the influence of a wound treatment on wound surface area and qualitative bacteriology (Bischofberger et al., 2011; Bischofberger et al., 2013; Kelleher et al., 2015). Some studies include or focus on a histological evaluation of the wound and evolution of growth factors (Monteiro et al., 2009, Broeckx et al., 2015; Bischofberger et al., 2016). However in our study, we monitored the effect of NPWT on wound dimensions (surface area, circumference, maximum depth and volume), bacterial load, limb swelling, wound temperature, clinical appearance, histological inflammation and repair scores, immunohistochemical presence of B-cells, T-cells, macrophages, endothelium and myofibroblasts and the evolution of growth factors TGF-β1 and TGF-β3. All these parameters were monitored to acquire a full impression on the overall effect of NPWT on the wound healing. We first tested NPWT on acute non-inoculated wounds (chapter 6.1) as is common in equine wound healing studies. However, in chapter 6.2, we expanded our study to inoculated wounds to gain more information on the impact of NPWT in a more real life situation.

Inoculated wound model

In chapter 6.2, we used a modified version of the wound model used in chapter 6.1, to test the effect of NPWT on second intention healing of contaminated wounds at the equine distal limb. The surface area and shape were the same as the wound model used in chapter 6.1, but this time the wounds were inoculated with two common equine wound pathogens: *Pseudomonas aeruginosa* and *Staphylococcus aureus* (Westgate et al., 2011). The

inoculation procedure was based on the method used in chapter 5 to inoculate the wounds in the *ex vivo* model. The bacteria were disolved in a stock suspension, which enabled a standardization of the number of applied bacteria based on densitometry. The number of bacteria was confirmed afterwards by quantitative bacteriology of the stock suspension. Additionally, during and after the inoculation procedure of the *in vivo* study (chapter 6.2) swabs were taken for quantitative bacteriology to ascertain a successful contamination of the wounds. The bacterial stock suspension was applied on a sterile gauze, because of the vertical surface of the wounds at the distal limb. In this way, the bacterial suspension would not run off the wound.

As mentioned in chapter 6.2, our inoculated wound model can also be considered as an infected wound model, since quantitative swabs indicated before commencement of the treatments a mean of 6.5 x 10^5 CFU per swab. So based on the results of chapter 4, were we found that a value of 1.2 x 10^5 CFU per swab corresponds with the critical value of > 1 x 10^5 CFU per gram of tissue for the quantitative biopsy, all but 2 wounds were infected based on bacterial load. Moreover, on the clinical assessment before commencement of the treatments (day 0), clinical signs (yellow discoloured granulation tissue, red granulation tissue, purulent exudate, friable granulation tissue) indicated an inflammation of the wounds. Associated with the heavy bacterial load as found with the quantitative swabs, the inflammation was probably due to infection.

An inoculated wound model enables the researcher to achieve a predicable level of contamination in a wound. This is useful to, for example, test different wound treatments and their effect on bacterial load without having the huge variation in bacterial species or number of bacteria in real life wounds. This model could also be used to test the influence of different bacterial levels or synergy between different bacterial species on wound healing. To the authors' knowledge, only one research group also used a contaminated equine wound model to test the effect of treatments on second intention wound healing (Bischofberger et al., 2011; Bischofberger et al., 2013; Bischofberger et al., 2016). This research group used faeces to inoculate wounds. However, the amount and species of micro-organisms in faeces is not standardized, creating a large variation in the wound contamination. Therefore, in our study (chapter 6.2), a standardized inoculation protocol was chosen with two repeated applications of a bacterial stock suspension of two common equine wound pathogens. A standardized inoculation protocol and thoroughly describing the applied methods enhances the repeatability of the procedure and thus the experiment. It also facilitates comparisons of different studies, and pooling of data of different studies with comparable methods.

In the first two studies of Bischofberger et al. (2011; 2013), only qualitative bacteriology was employed to verify the contamination of the wounds. Moreover, instead of a follow-up of the bacterial load over time, only qualitative bacteriology was performed to investigate the effect of manuka honey on the wound contamination. Thus, an evaluation of the effect of Manuka honey on bacterial load was not possible in these two studies. Moreover, the effectiveness of their inoculation procedure could not be verified. In the latest study of Bischofberger et al. (2016), quantitative bacteriology was employed to verify the inoculation procedure and to monitor the bacterial load over time. In this study quantitative bacteriology was performed on a tissue biopsy, the golden standard to follow-up bacterial load in a wound. However, because of the invasive nature of this method, Bischofberger et al. (2016) limited their sample moments to four time points. Additionally, the biopsies were taken each time from a different wound, but all designated to the same treatment group. Since the inoculation procedure was only verified in one wound and the follow-up of the bacterial load was each time performed on different wounds, a real evolution of the bacterial load over time of the wounds was not acquired. Based on the results of chapter 4, we chose quantitative bacteriology of a swab for the *in vivo* study to monitor the bacterial load over time and to verify the effectiveness of our inoculation procedure. This quantitative swab enabled us to monitor the bacterial load on regular time points (14 in total) and to perform repeated measurements on the same wound without interfering with the wound healing. Therefore, a clear overview of the evolution of bacterial load was obtained.

Comparison of negative pressure wound therapy for treating acute versus contaminated wounds

In our acute non-inoculated wound model (chapter 6.1), less wound retraction was seen for NPWT wounds compared to REF wounds in the treatment period (days 0-9) (Fig 1a). During the early period after treatment (days 14-29) NPWT wounds still had a smaller surface area compared to REF wounds (Fig. 1a). Since the collagen orientation was not significantly different between the two treatments, the less pronounced retraction in NPWT wounds is probably due to NPWT's mechanisms. NPWT keeps the wound edges physically together by a combination of the adhesive foil applied over the wound and the negative pressure applied to the wound bed. This theory is further reinforced by the absence of a significant difference in myofibroblasts between the two treatments in the acute non-inoculated wounds. In the study on our inoculated wound model (chapter 6.2), a larger wound surface area was also seen for REF wounds compared to NPWT wounds in the treatment period (days 0-6) and early period after treatment (days 9-29) (Fig. 1b), but the difference was not statistically significant. Possibly the variation of data on surface area was too large in the inoculated wounds for the differences to be significant. Additionally, the inoculated wounds were older

and thus already partially retraced when the treatment commenced compared to the acute non-inoculated wounds (4 days vs. 1 day). The acute non-inoculated wounds displayed a mean surface area of approximately 12 cm² before commencement of the study compared to a mean surface area of 13.5 cm² for the inoculated wounds. The presence of bacteria in the inoculated wounds probably also stimulated wound retraction, partially counteracting the treatment effect on the wound surface area (Bischofberger et al., 2013). Another possible explanation for the results not being significant in inoculated wounds, is the difference in length of the treatment period between the two studies. In the study on the acute non-inoculated wounds the treatment period included days 0 to 9, while in the study on inoculated wounds the treatment period included days 0 to 6 (Fig. 1). Therefore, more data points were present in the treatment period for the acute non-inoculated wounds, so significant differences are more easily detected.

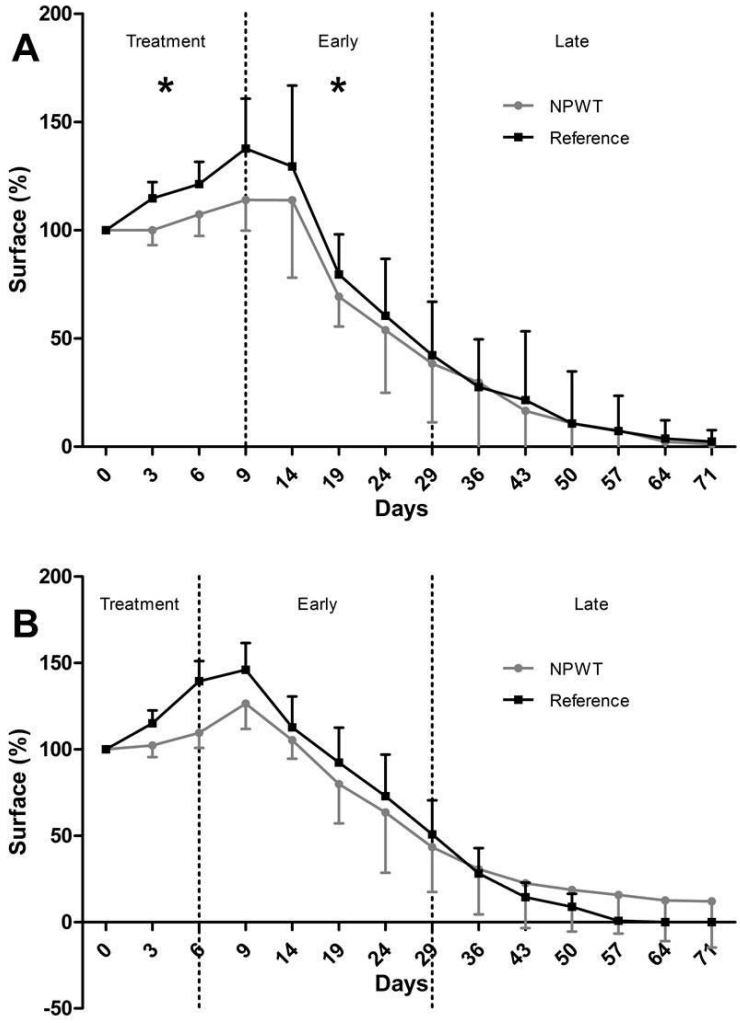

Figure 1. The evolution of the wound surface area over time for both treatments in percentages (mean + or - SD) for the study on acute wounds (A) and the study on inpculated wounds (B).

(A) In the study on acute wounds, the follow-up period was divided into the treatment period (day 0 until 9: Treatment), the early period after treatment (day 14 until 29: Early) and late period after treatment (day 36 until 71: Late). *: the mean surface area for this time period differed significantly between the treatment groups ($P < 0.05$). NPWT: negative pressure wound therapy using a standard polyurethane foam. Reference: Ca-alginate dressings. (B) In the study on inoculated wounds, the follow-up period was divided into the treatment period (day 0 until 6: Treatment), the early period after treatment (day 9 until 29: Early) and late period after treatment (day 36 until 71: Late). NPWT: negative pressure wound therapy using a silver impregnated polyurethane foam. reference: silver Ca-alginate dressings.

In the study on the acute non-inoculated wound model (chapter 6.1), NPWT wounds were significantly deeper during the treatment period and early period after treatment compared to REF wounds (Fig. 2a). Additionally, in the treatment period NPWT limbs were significantly more swollen compared to REF limbs. This swelling in the treatment period probably caused a more pronounced increase of wound maximum depth for NPWT compared to REF wounds (Fig. 2a). Moreover, the more rapid decrease of maximum depth in the early period after treatment for REF wounds indicated that the calcium alginate dressings induced a more rapid formation of granulation tissue compared to NPWT. In the inoculated wound model (chapter 6.2), NPWT wounds also seemed deeper in the treatment period and the early period after treatment, albeit less pronounced than in the study on the acute wound model (Fig. 2b). Indeed, these differences were not found to be significant after statistical analysis. Additionally, in the inoculated wounds no significant difference was found in the swelling of the limbs between the two treatments, concurring with the results on the maximum depth. An explanation for the less pronounced differences in the inoculated compared to the acute non-inoculated wounds is, as already mentioned in the previous paragraph, the difference in duration of the wounds at the commencement of the study (4 days vs. 1 day). Since the inoculated wounds were older, they were already partially filled with granulation tissue. Thus, less granulation tissue needs to fill up the rest of the defect in inoculated versus acute non-contamianted wounds, resulting a less pronounced treatment effect.

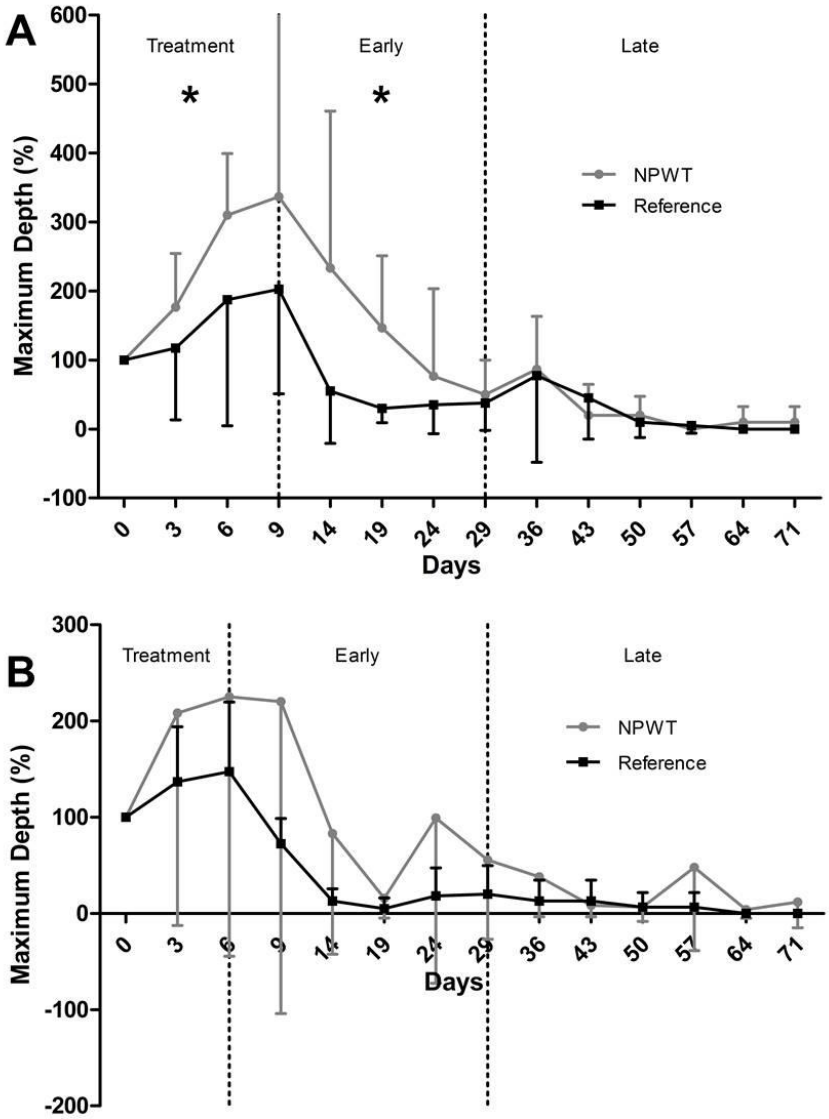

Figure 2. The evolution of wound maximum depth over time for both treatments in percentages (mean + or - SD) for the study on acute wounds (A) and the study on inoculated wounds (B).

(A) In the study on acute wounds, the follow-up period was divided into the treatment period (day 0 until 9: Treatment), the early period after treatment (day 14 until 29: Early) and late period after treatment (day 36 until 71: Late). *: the mean maximum depth for this time period differed significantly between the treatment groups ($P < 0.05$). NPWT: negative pressure wound therapy using a standard polyurethane foam. Reference: Ca-alginate dressings. (B) In the study on inoculated wounds, the follow-up period was divided into the treatment period (day 0 until 6: Treatment), the early period after treatment (day 9 until 29: Early) and late period after treatment (day 36 until 71: Late). NPWT: negative pressure wound therapy using a silver impregnated polyurethane foam. Referehce: silver Ca-alginate dressings.

In contrast to the acute non-inoculated wounds (chapter 6.1), the bacterial load of both NPWT and REF wounds in chapter 6.2 was not stable over time. The bacterial load in the inoculated wounds was significantly higher in the treatment period compared to the early and late period after treatment for both therapies. Since the statistical analysis was not performed on the individual time points but on a period of time, statistical differences between individual time points were not investigated. However, a decrease of bacterial load for each time point in the treatment period was seen for both treatments. This probably indicates an antibacterial effect of the combination of the silver ions of the treatments and the wound cleansing performed during bandage changes. During the early period after treatment, bacterial load

for both treatments still decreased steadily over time. For REF wounds the bacterial load started to increase again in the late period after treatment compared to the early period after treatment, though not significantly different compared to the NPWT group. This increase could be coincidental or as a result of the increasing difficulty to swab the wound when the surface area decreases. In the acute non-inoculated wound model (chapter 6.1), the bacterial load remained relatively stable with an approximate mean load of 10^3 CFU/swab. In the inoculated wound model (chapter 6.2), a mean load of approximately 10^6 CFU/swab was found at the beginning of the study, which declined to a mean load of approximately 10^3 CFU /swab at the end of the study. This study clearly shows that a healing wound is not aseptic. This is also not necessary to progress to a closed wound as long as the immune system of the host can overcome the bacterial load (Edwards and Harding, 2004).

In both the studies on acute non-inoculated wounds and inoculated wounds (Chapter 6.1 and 6.2), the temperature of the wounds did not significantly differ between the two treatments. However, for the acute wounds the temperature in the treatment period was significantly higher than the temperature in the early period after treatment, whereas in the inoculated wounds the temperature in the treatment period was significantly higher compared to the temperature in the late period after treatment (Fig. 3). In the acute non-inoculated wounds, there was first an increase in temperature in the treatment period followed by a decrease, while in the inoculated wound there was only a steadily decrease of the temperature over time. This difference can be explained by the difference in duration of the acute non-inoculated and inoculated wounds at the beginning of the studies. The wounds in the inoculated model were older (4 days vs. 1 day), and thus the inflammation response was already more profoundly present, while in the in the acute wounds this was still in a starting phase. Moreover, the presence of a higher bacterial load in the inoculated wounds additionally stimulated the inflammatory response, probably causing a higher temperature, and indirectly blood flow, of the wounds. Therefore, in the study on the acute non-inoculated wounds the temperature probably increased with the increase of the inflammatory response, while with the inoculated wounds, the study started on the peak of the inflammatory response, which then steadily decreased over time.

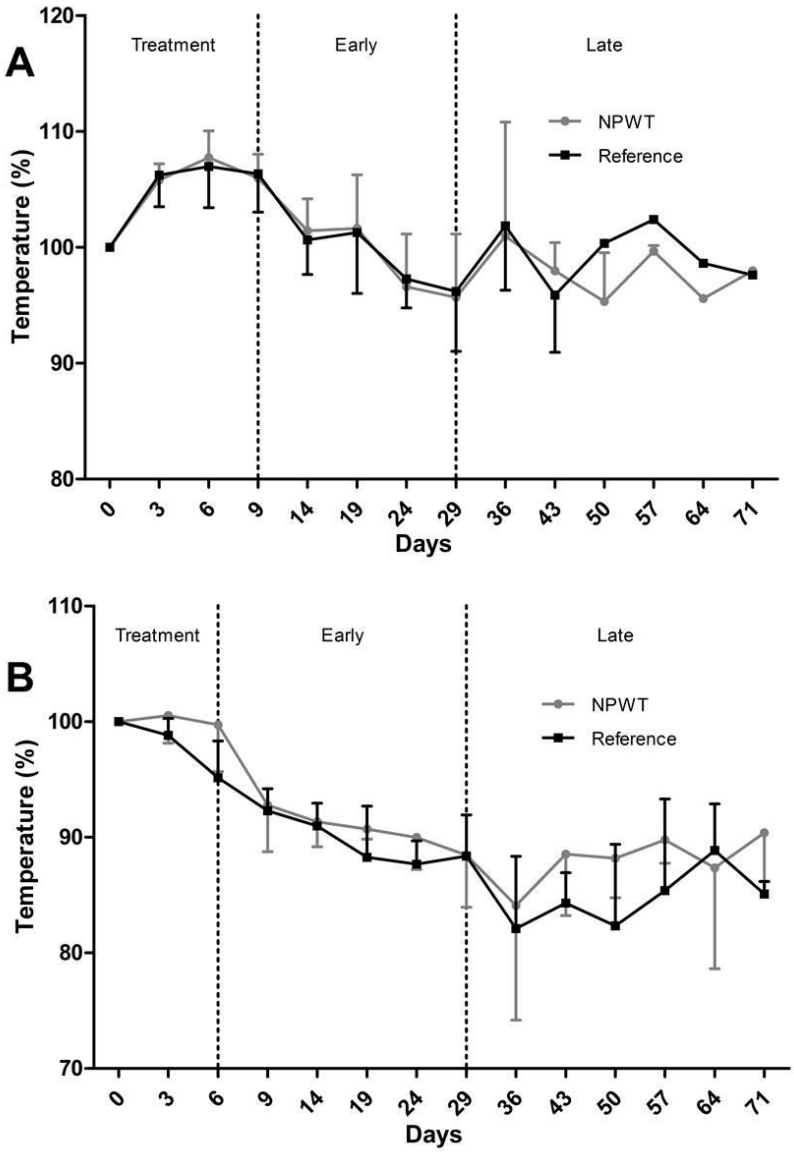

Figure 3. The evolution of wound temperature (an indirect measure for blood flow) over time for both treatments in percentages (mean + or - SD) for the study on acute wounds (A) and the study on inoculated wounds (B). (A) In the study on acute wounds, the follow-up period was divided into the treatment period (day 0 until 9: Treatment), the early period after treatment (day 14 until 29: Early) and late period after treatment (day 36 until 71: Late). NPWT: negative pressure wound therapy using a standard polyurethane foam. Reference: Ca-alginate dressings. (B) In the study on inoculated wounds, the follow-up period was divided into the treatment period (day 0 until 6: Treatment), the early period after treatment (day 9 until 29: Early) and late period after treatment (day 36 until 71: Late). NPWT: negative pressure wound therapy using a silver impregnated polyurethane foam. Reference: silver Ca-alginate dressings.

When comparing the histology between our two *in vivo* studies on NPWT (chapter 6.1 and 6.2), significant differences between the two treatments were only seen in the study on acute non-inoculated wounds. Probably, the presence of the higher bacterial load in the inoculated wounds influenced the inflammatory response to such an extent that treatment effects were no longer relevant. Subjectively, the author had the impressions that the granulation tissue of the latest biopsies (day 29) in the acute non-inoculated wounds was more organized and mature based on the parameters of the histological repair score, than the granulations tissue of the latest biopsies in the inoculated wounds. However, this was not confirmed with a statistical test.

Both in the acute non-inoculated and inoculated wounds there was a significant difference between NPWT and REF treatment in certain clinical parameters. In chapter 6.1, REF wounds were significantly more likely to present oedematous granulation tissue and an unpleasant odour. In chapter 6.2, REF wounds were significantly more likely to present oedematous granulation tissue and purulent exudate. In contrast, NPWT wounds were significantly more likely to present sanguineous exudate and red granulation tissue. These clinical differences between the two treatments in both studies could not be confirmed histologically. Probably, the number of samples for histology was too low or the used scoring systems were not sensitive enough to detect the differences.

Negative pressure wound therapy in veterinary medicine

In veterinary medicine, only one other study investigated the effect of NPWT on second intention healing wounds in a controlled manner (Demaria et al., 2011). This study was performed on dogs and also used an acute wound model (8cm²) but on the antebrachium. Demaria et al. (2011) investigated the influence of NPWT on wound surface area, the first appearance of granulation tissue, quality of granulation tissue and histological inflammation and repair scores. They also performed semi-quantitative bacteriology. The study design of Demaria et al. (2011) deviated from our set up by the use of different primary dressings with NPWT and reference wounds, in duration of NPWT and wound location. They used either a gauze or a standard polyurethane foam as primary dressing with NPWT. As a reference dressing they used a hydrophilic polyurethane foam during the entire study period. Both treatments were applied for the entire study period of 21 days, while in our study the NPWT and calcium alginate dressings were applied for only 9 days until the wounds were granulated, as is currently done in equine practice. Demaria et al. (2011) found a faster appearance of granulation tissue in the NPWT wounds compared to the reference wounds and the tissue was smoother and less exuberant compared to the reference wounds. However, the wound surface area in their study was larger for NPWT wounds compared to reference wounds from day 7. There was also a higher bacterial load in NPWT wounds on day 7 and a lower histological acute inflammation score. This study is hard to compare to our studies because of the differences in study design. However, based on this study we only applied NPWT until the wounds were fully granulated, because Demaria et al. (2011) mentioned that NPWT possibly intervenes with epithelialization of the wound.

After performing our studies, the manufacturer of the used NPWT system suggested that NPWT could enhance epithelialization by reducing the foam size at each bandage change. In this way, the migrating epithelium could advance over the granulation bed without hindrance from the foam. However, horses have the tendency to develop exuberant granulation tissue

at their distal limbs (Theoret and Wilmink, 2008). Therefore, when the wound is fully granulated and displays healthy granulation tissue, the goal is to slow down the fibroplasia and inflammatory response and to stimulate the epithelialization. Since NPWT is known in men to stimulate granulation tissue formation (Mouës et al., 2011), this is not the preferred treatment at this stage. The manufacturer suggested that we could use a polyvinyl alcohol foam as primary dressing once granulation tissue is formed, as this dressing stimulates the granulation tissue formation to a lesser extent and provides a smooth granulation bed in the wound. However, no controlled study exists on the use of polyvinyl alcohol foams with NPWT in human or veterinary medicine. Thus, these suggestions are based on empirical findings and expert opinions. It would be therefore interesting to test these statements in a future study.

The bandage material used with NPWT is accommodated to the application in humans. This has some practical implications when applying a NPWT bandage in veterinary medicine, mainly concerning the maintenance of the airtight seal. The skin around the wound has to be thoroughly shaved and degreased in animals to increase the adhesion between the skin and the foil. The skin is best shaved 24 hours in advance to prevent blood from little cuts in the skin accumulating beneath the foil. The airtight seal can also be enhanced by applying adhesive spray on the skin around the wound, or two-sided adhesive gel strips. The manufacturer also suggested the use of adhesive hydrocolloid dressings around the wound to increase the seal between the foil and the wound surroundings and protect the skin. Because of these difficulties to maintain the airtight seal, NPWT bandages in animals are more likely to display little leaks, necessitating additional bandages changes or application of extra adhesive foil. This increases the costs of NPWT treatment in animals. In general, NPWT is considerably more expensive than REF therapy in veterinary medicine. In our institution NPWT costs an owner about 8 times as much as treatment with (silver) calcium alginate dressings. However, this does not include working hours or costs for sedation of the horse. In our in vivo studies the amount of bandage changes were the same for the REF and NPWT treatment to ensure uniformity between the treatments and standardization of the sample moments. In human medicine, there has been reported that NPWT is more cost effective than the standard treatments because of less bandage changes and a shorter hospitalisation period (Birke-Sorensen et al., 2011; Vig et al., 2011; Vassallo and Formosa, 2015). However, to our experience with equine clinical cases, NPWT does not require less bandage changes. On the contrary because of the difficulties to maintain the airtight seal, additional bandage revisions are sometimes necessary to ensure the preservation of the negative pressure. However, to confirm this objectively, a randomized prospective clinical study should be performed, which compares NPWT to a standard wound treatment. On other

hand, it can indeed be questioned if a trial on a non-promising treatment technique purely to confirm this statement is worth the resources and animals. Since NPWT is costlier than calcium alginate therapy, NPWT should drastically reduce the overall healing time, reduce complications or improve the quality of the scar tissue to be cost effective. However, these findings were not seen in our *in vivo* studies, making the routine use of NPWT for equine distal limb wounds less interesting. If NPWT could be used to treat wounds which otherwise not respond to treatment, this would also justify the additional costs.

Another practical problem in veterinary medicine is the immobilization of the animals during the NPWT treatment or during the bandage changes. Currently, mobile systems can be used (V.A.C. freedom or actiV.A.C, KCI medical) or the tubing can be adapted so the animals can move freely (Gemeinhardt and Molnar, 2005; Demaria et al., 2011). However, when the tubing is adapted, the build-in alarm system no longer works and the mobile systems are often not as powerful as the solid systems. This is especially a problem in horses, where traumatic wounds are often larger and more complex than in small animals. Therefore, horses are typically tethered at both sides of their heads to minimize their range of movement during the therapy, which however decreases their well-being. Additionally, during the bandage changes of NPWT, animals have to be sedated for safety and pain management reasons (Demaria et al., 2011; Stanley et al., 2013). This again increases the costs of NPWT treatment in animals compared to humans.

Negative pressure wound therapy in human medicine

In human medicine, NPWT has been shown to be beneficial for several wound types compared to standard therapy. Good results have been acquired for the treatment of diabetic foot ulcers, pressure ulcers, open fractures, fasciotomy wounds and burn wounds with NPWT (Krug et al., 2011; Vig et al., 2011; Mouës et al., 2011). Additionally, NPWT also seems to increase graft take with split thickness skin grafts (Krug et al., 2011). However, studies which compare NPWT to calcium alginate dressing are scant. A study of Monsen et al. (2015), reported a significantly faster full skin epithelialization for NPWT treated groin wounds compared to the calcium alginate treated wounds. Wound volume or depth measurements were not performed in this study. Qualitative bacteriology was performed, but no significant difference was found between the two treatments. Another study of Vassallo and Formosa (2015) comparing NPWT to calcium alginate dressings to treat diabetic ulcers, found that the reduction in surface area and wound depth from beginning to the end of the study was significantly larger for NPWT wounds compared to the calcium alginate treated wounds. The wound surface area of NPWT wounds reduced with a mean of 3.57 cm^2 compared to a mean of 1.09 cm^2 for the calcium alginate wounds, while the maximum depth

reduced with a mean of 0.68 cm vs. 0.18 cm with NPWT and calcium alginate respectively. Both treatments significantly reduced wound surface area and maximum wound depth over time. However, the study of Vassallo and Formosa (2015) did not mention the duration of follow up period of the wounds for the two treatments. The article also does not mention clearly if the treatments were applied until wound closure or until healthy granulation tissue was achieved. Thus, the results of this study should be interpreted carefully.

The results of treatment with NPWT in human medicine are more optimistic than the results acquired in our *in vivo* studies (chapter 6.1 and chapter 6.2). In human medicine, NPWT is often applied until a healthy granulation bed is formed and the wound can be successfully closed by delayed closure or with skin grafts (Krug et al., 2011; Vig et al., 2011). If this is not possible, NPWT can be used to let the wounds heal further by second intention (Krug et al., 2011, Vig et al., 2011). In equine medicine, NPWT is not used for these aims, because of the practical objections, costs, and the chance on exuberant granulation tissue. Moreover, delayed closure is often not possible because of massive tissue loss and skin retraction. Therefore, equine wounds are more frequently left to heal by second intention (Stashak, 2008b; Schumacher and Wilmink, 2008). Therefore, the pursued end goal with NPWT treatment differs between human and equine medicine, possibly explaining our less optimistic results. Additionally, our studies used an experimental model with rather 'young' wounds (1 to 4 days old), whereas in human medicine NPWT is often used to treat chronic wounds, resistant to treatment for several days to even one year (Vig et al., 2011). In horses however, older non healing wounds often display exuberant granulation tissue rather than a lack of granulation tissue, requiring a different treatment approach (Wilmink, 2014). Nonetheless, acute traumatic wounds at the distal limb in horses display similarities with chronic non healing wounds in humans because of the sluggish and inefficient inflammatory response (Wilmink, 2014).

Future perspectives for negative pressure wound therapy in equine medicine

For future research, it would be interesting to have a controlled study on the use of NPWT to enhance skin graft take in horses. The authors have good experiences with NPWT for this application, concurrent with the case reports in the literature (Rijkenhuizen et al., 2005; Jordana et al., 2011). However, no objective controlled studies have been performed in horses. In dogs, Stanley et al. (2013) reported superior graft take of full thickness skin grafts with NPWT. It would be interesting to see if these results also apply on horses. An additional interesting field of research would be NPWT with instillation. In human medicine, this technique seems to yield promising results compared to purely NPWT (Rycerz et al., 2013). The rate of granulation tissue is faster and bacteria are more effectively removed compared

to standard wound irrigation during the NPWT bandage changes (Rycerz et al., 2013). This technique could thus potentially improve wound healing in contaminated equine distal limb wounds. However, the authors foresee some practical difficulties in applying this technique to horses. For example, when the fluid is instilled and left to soak, it is plausible that the fluid starts to run between the foil and the skin, breaking the airtight seal. This effect would be worse on vertical surfaces such as the distal limb in horses. Knowing the difficulties to maintain the vacuum in horses, this is an area for concern. Additionally, when using NPWT with instillation, the negative pressure has to be restarted after each instillation cycle. In humans, intermittent application of the negative pressure is known to cause pain and discomfort (Birke-Sorensen et al., 2011). Therefore, it can be expected that the restart of the negative pressure would cause discomfort and resistance in horses. Another interesting topic to investigate is the use of the polyvinyl alcohol foam dressing with NPWT in horses. To the authors' knowledge no *in vivo* studies have been performed on the effect of these dressing on wound healing in human or veterinary medicine. Additionally, it would be interesting to test the potential of NPWT to decrease surgical site infection after laparotomy.

Conclusions

To conclude, compared to (silver) calcium alginate dressings, the effects attributed to NPWT in human medicine such as the stimulation of granulation tissue, increase in blood flow and stimulation of angiogenesis could not be confirmed in our experimental *in vivo* studies. However, NPWT was found to reduce wound surface area and modulate the inflammatory response in experimental acute non-inoculated wounds. Additionally, NPWT using a silver impregnated polyurethane foam also reduced bacterial load when treating experimental inoculated wounds, albeit not significantly better than silver calcium alginate dressings. The overall healing time was also not significantly reduced with NPWT. Therefore, NPWT does not seem to have an added value over the use of the standard treatment for acute and contaminated second intention healing wounds in the equine distal limb during the fibroproliferative phase, namely (silver) calcium alginate dressings. Additionally, during this PhD study 3 wound models were developed. An *ex vivo* wound model, which permits preliminary testing of the antibacterial activity of wound dressings and 2 *in vivo* wound models, one acute non-inoculated and one inoculated model. These *in vivo* models are more realistic representations of real life traumatic wounds than the most common wound models currently used in equine medicine and therefore enable more clinically relevant conclusions to be drawn about treatments for real life wounds. This PhD study also showed that the laser beam camera was a reliable and user friendly technique to follow up wound dimensions, so this technique is now further used in other research projects and for equine patients. Moreover, the quantitative swab proved to be a good non-invasive alternative to a

quantitative biopsy to monitor bacterial load in open wounds, and can thus be used for research and clinical purposes.

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SUMMARY

Traumatic wounds are very common in horses and are a cause of noteworthy solicitude for both owner and equine practitioner. These wounds are often left open to heal by second intention because of massive tissue loss, high skin tension and heavy bacterial contamination. Second intention healing is a time consuming process and the esthetical outcome is often unsatisfactory. Moreover, when traumatic wounds are present at the distal limbs, their healing is often impeded because of an initial sluggish inflammatory response follow by chronic wound inflammation leading to excessive granulation tissue formation. Negative pressure wound therapy (NPWT) is being used successfully in human medicine to treat a wide variety of wounds and it could aid to overcome typical problems seen with second intention wound healing at the equine distal limb. However, research on the use of NPWT in horses is scarce, making veterinarians cautious in using this technique to promote wound healing in horses.

Chapter 1 of this PhD study provides an overview of the general wound healing process in horses and the difficulties seen with second intention wound healing at the equine distal limb. Moreover, a guideline is presented to treat second intention healing wounds in horses based on the current literature. The latest developments in equine wound management are described and an overview is given of old wound healing techniques which regained renewed interest. Additionally, a more thorough explanation is given on the history of negative pressure wound therapy, its mechanisms of action, indications and contraindications in human medicine and how to apply a NPWT bandage in a horse. At the end of this chapter, the potential value of NPWT to promote second intention wound healing is reviewed, with an emphasis on the difficult healing of open wounds at the equine distal limb.

The scientific aims of this work are presented in **chapter 2**. The main goals were to refine the techniques needed to perform *in vivo* wound healing studies, such as methods needed for measuring wound dimensions (chapter 3) and monitoring bacterial load in open wounds (chapter 4). Additionally, we wanted to test different primary dressings used with NPWT for their antibacterial activity in an *ex vivo* wound model (chapter 5). However, the most important goal of this PhD study was to investigate the added value of NPWT on second intention healing of acute and contaminated wounds in the equine distal limb (chapter 6.1 and chapter 6.2).

Chapter 3 describes a validation of two techniques for measuring wound dimensions in open wounds in horses. The accuracy, precision, inter- and intra-operator reliability of a new laser beam (LB) wound camera and a digital photoplanimetry-based (DPB) method were compared to each other. Forty-one wounds were created on equine cadavers. The area,

circumference, maximum depth and volume of each wound were measured four times with both techniques by two operators. A silicone cast was made of each wound and served as the reference standard to measure the wound dimensions. The DPB method had a higher accuracy and precision in determining wound volume compared with the LB camera, which had a higher accuracy in determining wound area and maximum depth, and a better precision in determining area and circumference. The LB camera also had a significantly higher overall inter-operator reliability for measuring wound area, circumference and volume. In contrast, the DPB method had poor intra-operator reliability for the wound circumference. The LB camera was more user-friendly than the DPB method. It was concluded that the LB wound camera was the better objective method to assess the dimensions of wounds in horses, despite its poorer performance for the measurement of wound volume.

In **Chapter 4** different techniques were assessed to monitor bacterial load in second intention healing wounds in horses and the influence of biofilms on quantitative bacteriology was investigated. In fifty second intention healing wounds, a clinical assessment, quantitative swab, semi-quantitative swab, and a swab for cytology were compared to a quantitative tissue biopsy (reference standard). Part of the biopsy was examined histologically for evidence of a biofilm. A high correlation ($P < 0.001$ $r = 0.747$) was shown between the outcome of the quantitative swabs and the quantitative biopsies. These were linearly related ($P < 0.001$) by the regression function $Y = 1.121 + 0.846 X$ ($r^2 = 0.56$), where Y represents the log colony-forming units (CFU) of the quantitative biopsy and X represents the log CFU of the quantitative swabs. The semi-quantitative swabs showed a significant but only moderate correlation with the quantitative biopsies ($P < 0.001$ $\rho = 0.524$). Higher white blood cell counts for cytology were significantly associated with lower log CFU in the wounds ($P = 0.02$). Wounds with black granulation tissue showed significantly higher log CFU ($P = 0.003$). Samples with biofilms did not give a higher bacteriological count after a vortex and sonication protocol was performed to release bacteria from the biofilm. It was concluded that the quantitative swab seems a good non-invasive alternative to the quantitative biopsy to determine bacterial load in open wounds in horses.

Chapter 5 reports the comparison of the antibacterial effect of negative pressure wound therapy (NPWT) using three different foams to a standard antibacterial dressing in an equine perfused *ex vivo* wound model. An abdominal musculocutaneous flap was collected from six equine cadavers. Four circular wounds of 5 cm diameter were created per flap and were inoculated with methicillin resistant *Staphylococcus aureus* (MRSA) and *Pseudomonas aeruginosa*. After an incubation period, the wounds were assigned to one of following four treatment groups: (1) NPWT using a silver impregnated polyurethane foam (NPWT-AgPU), (2) NPWT using a normal polyurethane foam (NPWT-PU), (3) NPWT using a polyvinyl

alcohol foam (NPWT-PVA) or (4) reference treatment (R) using a non-adherent antimicrobial dressing with polyhexamethylene biguanide without NPWT. An 8 mm punch biopsy was obtained from each wound before application of the treatments (T0) and then every 6 hours during the 24 hour treatment protocol (T6, T12, T18, T24) to calculate the bacterial load. For *P. aeruginosa*, NPWT-PVA resulted in a significantly lower bacterial load from T6 to T24 compared to the other three treatments. The bacterial load with NPWT-AgPU was lower than with R and NPWT-PU from T6 to T24 but only significantly so at T12. For MRSA, the bacterial load with NPWT-PVA was significantly lower compared to the other treatments from time point T6 on. It was concluded that NPWT-PVA gave the greatest reduction in bacterial load, but this dressing should be tested *in vivo* to assess its influence on wound healing.

In **Chapter 6** the added value of NPWT on second intention healing of equine distal limb wounds was assessed using the techniques described in the previous chapters. The clinical relevance of NPWT was first tested on an acute non-inoculated wound model (chapter 6.1), followed by an inoculated wound model (chapter 6.2).

The added value NPWT was assessed on second intention healing of acute equine distal limb wounds compared to Ca-alginate dressings in **chapter 6.1**. Two circular 3.5 cm diameter wounds were created on both metacarpi in 5 horses. In the first 9 days, one limb was treated with NPWT, while the contralateral limb was treated with Ca-alginate dressings (reference). From day 9 to 71 both limbs were kept under bandage with hydrophilic polyurethane foam dressings. Over the course of healing, wound dimensions were measured, swabs were taken for quantitative bacteriology and blood flow was indirectly measured using a thermographic camera. At 5 time points, an 8 mm punch biopsy was taken for growth factor analysis and histological scoring. The wound area of NPWT wounds was significantly smaller compared to the reference wounds during the 9-days treatment period ($P = 0.005$) and in the early period after treatment ($P = 0.003$), but not in the late period after treatment. The maximum depth of NPWT treated wounds was significantly larger compared to reference wounds during the treatment period ($P = 0.007$) and the early period after treatment ($P = 0.029$), but not in the late period after treatment. The reference wounds had a significantly higher ($P = 0.044$) histological score for acute inflammation compared to NPWT-treated wounds. However, in NPWT wounds, significantly ($P = 0.008$) more macrophages were present. There was no significant difference in histological repair scores, growth factor concentrations, nor in the presence of B-cells, T-cells, myofibroblasts and neovascularisation. Based on the results, no real advantage could be detected in the use of NPWT over Ca-alginate dressings for the treatment of acute distal limb wounds in horses.

In **chapter 6.2,** the added value of NPWT was assessed on second intention healing of contaminated equine distal limb wounds compared to silver Ca-alginate dressings. Two circular 3.5 cm diameter wounds were created on both metacarpi in 5 horses. Each wound was inoculated with two common equine wound pathogens: *Staphylococcus aureus* and *Pseudomonas aeruginosa*. In the first 6 days, one limb was treated with NPWT, while the contralateral limb was treated with silver calcium alginate dressings (reference). From day 6 to 71, both limbs were kept under bandage with hydrophilic polyurethane foam dressings. Over the course of healing, wound dimensions were measured, swabs were taken for quantitative bacteriology and blood flow was indirectly measured using a thermographic camera. On 5 time points, an 8 mm punch biopsy was taken for growth factor analysis and histological scoring. No significant difference between the two treatments could be detected for any of the wound dimensions (surface area, maximum depth and wound volume). There were also no significant differences in bacterial load, wound temperature (hence blood flow) and limb swelling between NPWT and reference wounds. There was no significant difference in histological inflammation and repair scores, nor in the presence of B-cells, T-cells, macrophages, myofibroblasts and neovascularisation. The concentration of the growth factors transforming growth factor β1 and β3 was not significantly different between the two treatments. Based on the results, no real advantage could be detected in the use of NPWT over silver Ca-alginate dressings for treatment of contaminated distal limb wounds in horses.

Chapter 7 places the findings of this PhD study in a general context, describes the main conclusions and looks at the future research possibilities. This PhD study provided insight on the different effects NPWT on an acute non-inoculated wound model and an inoculated wound model in the horse. In the future, the potential of NPWT to enhance the survival of skin grafts in horses should be tested. Moreover, different primary dressings or other study designs can be used to get a more profound knowledge on the effect of NPWT on second intention healing in horses. Additionally, refinement of the used wound models remains necessary, including a refinement of the used methods to monitor wound healing parameters.

SAMENVATTING

Traumatische wonden zijn een veelvoorkomend probleem bij paarden. Ze zorgen dan ook voor aanzienlijke kopzorgen bij paardeneigenaren en praktiserende paardendierenartsen. Traumatische wonden bij paarden vertonen vaak een zeer zware bacteriële contaminatie, een aanzienlijk verlies van weke delen en bijgevolg een hoge spanning op de omliggende huid waardoor deze vaak open worden gelaten om per secundam te helen. Helaas is het 'per secundam' helingsproces tijdrovend en laat de cosmetiek van het resulterende litteken vaak te wensen over. Als de wonde zich bovendien op het onderste lidmaat van het paard bevindt (onder de carpus of tarsus), wordt de heling extra vertraagd door een initieel inefficiënte ontstekingsreactie, gevolgd door een eerder chronische wondinflammatie die leidt tot de vorming van hypergranulatieweefsel welke inherent is aan deze locatie. Een mogelijke oplossing voor deze vertraagde wondheling ter hoogte van de distale ledematen is het gebruik negatieve druktherapie (NDT). NDT wordt succesvol gebruikt in de humane geneeskunde om diverse soorten wonden te behandelen en zou kunnen helpen om de typische problemen van per secundam wondheling aan het onderste lidmaat bij paarden te overwinnen. Er is echter nog maar bijzonder weinig onderzoek verricht naar NDT bij paarden, waardoor deze techniek in de paardengeneeskunde nauwelijks wordt toegepast om wondheling te stimuleren.

Hoofdstuk 1 van deze doctoraatsthesis geeft een overzicht van het algemene wondhelingsproces bij paarden en verduidelijkt de problemen die worden waargenomen bij 'per secundam' heling van wonden aan het onderste lidmaat bij paarden. Dit hoofdstuk geeft ook richtlijnen over hoe een 'per secundam helende' wonde bij het paard behandeld kan worden op basis van de huidige wetenschappelijke literatuur. Daarnaast worden de laatste ontwikkelingen in de wondbehandeling van paarden aangehaald en wordt een overzicht gegeven van oude wondbehandelingsmethoden die opnieuw in de aandacht zijn gekomen. Op een van deze technieken, namelijk NDT, wordt er verder ingegaan. De geschiedenis van NDT wordt beschreven, samen met de werkingsmechanismen, indicaties en contra-indicaties bij mensen. Er wordt ook beschreven hoe een NDT verband bij paarden wordt aangelegd. Op het einde van dit hoofdstuk wordt er verder ingegaan op het potentieel van NDT voor de behandeling van' per secundam' helende wonden bij paarden, met de nadruk op het gebruik van deze therapie voor de moeilijk helende open wonden aan het onderste lidmaat bij paarden.

De wetenschappelijk doelstellingen van dit werk worden besproken in **hoofdstuk 2**. Een van de doelstellingen was het verfijnen van de technieken die nodig zijn om *in vivo* wondhelingsstudies te verrichten, zoals een methode om de afmetingen van open wonden te meten (hoofdstuk 3) en om de bacteriële belasting in deze wonden na te gaan (hoofdstuk 4). Bijkomend werd op een *ex vivo* wondmodel het antibacteriële effect van verschillende

primaire dressings gebruikt bij NDT nagegaan (hoofdstuk 5). De hoofddoelstelling van deze doctoraatsthesis was echter het nagaan van de meerwaarde van NDT voor de behandeling van 'per secundam helende' acute en gecontamineerde wonden aan het onderste lidmaat bij het paard (hoofdstuk 6.1 en hoofdstuk 6.2).

In **hoofdstuk 3** wordt de validatie beschreven van twee technieken voor het meten van wonddimensies in open wonden bij paarden. De accuraatheid, precisie, inter- en intra-operator betrouwbaarheid van een nieuwe laser wond camera en een digitale fotoplanimetrie gebaseerde (DFB) methode werden met elkaar vergeleken. Er werden 41 wonden gemaakt op paardenkadavers. De oppervlakte, omtrek, maximale diepte en volume van elke wonde werd vier keer gemeten met beide technieken door twee operatoren. Siliconen afgietsels van de wonden werden gebruikt als referentie om de wonddimensies te bepalen. De DFB methode had een hogere accuraatheid en precisie voor het meten van de wondvolume in vergeleken met de laser wond camera. Deze laatste had op zijn beurt een betere accuraatheid voor het bepalen van het wondoppervlak en de maximale diepte en een betere precisie voor het bepalen van het wondoppervlak en de omtrek. De laser wond camera had bovendien een significant betere algemene inter-operator betrouwbaarheid voor het meten van het wondoppervlak, de omtrek en het volume. In tegenstelling bezat de DFB methode een slechte intra-operator betrouwbaarheid voor het meten van de wondomtrek. De laser wond camera was ook gebruiksvriendelijker dan de DFB methode. Er werd geconcludeerd dat de laser wond camera de betere objectieve methode was om wond dimensies bij paarden te beoordelen, ondanks de mindere prestaties van deze techniek om het wondvolume te meten.

In **hoofdstuk 4** werden verschillende technieken beoordeeld om de bacteriële belasting in 'per secundam' helende wonden bij paarden na te gaan, en werd de invloed van biofilms op de kwantitatieve bacteriologie onderzocht. In 50 per secundam helende worden werd een klinische beoordeling, kwantitatieve swab, semi-kwantitatieve swab, en een swab voor cytologie vergeleken met een kwantitatief weefsel biopt (de referentie standaard). Een deel van het weefsel biopt werd ook histologisch onderzocht op de aanwezigheid van een biofilm. Er werd een hoge correlatie ($P < 0,001$ $r = 0,747$) aangetoond tussen de uitkomst van de kwantitatieve swabs en de kwantitatieve biopsieën. Er was een lineair verband tussen deze twee methoden, beschreven door de regressie functie $Y = 1,121 + 0,846\ X$ ($r^2 = 0,56$), waarbij Y de log kolonie-vormende eenheden (KVE) van het kwantitatieve biopt voorstelt en X de log KVE van de kwantitatieve swab. De semi-kwantitatieve swabs vertoonden een significante maar matige correlatie met de kwantitatieve biopsieën ($P < 0,001$ $\rho = 0,524$). De swab voor cytologie en de parameters van de klinische beoordeling had geen voorspelende waarde om te bepalen of een wond geïnfecteerd was of niet. De waarden van de

bacteriologische telling in stalen met een biofilm waren niet hoger nadat er een vortex en sonicatie protocol was uitgevoerd om bacteriën te bevrijden uit de biofilm. Er werd geconcludeerd dat de kwantitatieve swab een goed niet-invasief alternatief is voor het kwantitatieve biopt om de bacteriële belasting in een open wond bij paarden na te gaan.

Hoofdstuk 5 vergelijkt het antibacteriële effect van NDT, waarbij drie verschillende sponzen gebruikt werden als primaire dressing, met een standaard antibacteriële dressing in een geperfuseerd *ex vivo* wond model voor het paard. Een abdominale spierhuid flap werd aseptisch gecollecteerd bij 6 paardenkadavers. Vier circulaire wonden van 5 cm diameter werden gecreëerd per flap en elke wonde werd geïnoculeerd met methicilline resistentie *Staphylococcus aureus* (MRSA) en *Pseudomonas aeruginosa*. Na een incubatieperiode werden de wonden toegewezen aan één van de 4 volgende behandelingsgroepen: (1) NDT met een zilver geïmpregneerde polyurethaan spons (NDT-AgPU), (2) NDT met een normale polyurethaan spons (NDT-PU), (3) NDT met een polyvinyl alcohol schuim (NDT-PVA) of (4) referentie behandeling (R) met een niet-adherente antimicrobiële dressing met polyhexamethyleen biguanide zonder NDT. Om de bacteriële belasting te berekenen werd er een 8 punch biopt genomen van elke wonde voor het aanbrengen van de behandelingen (T0) en vervolgens elke 6 uur gedurende het 24 uur durende behandelingsprotocol (T6, T12, T18, T24). Voor *P. aeruginosa* zorgde NDT-PVA voor een significant lagere bacteriële belasting van T6 tot T24 in vergelijking met de drie andere behandelingen. De bacteriële belasting bij NDT-AgPU was lager dan bij R en NDT-PU van T6 tot T24, maar dit was enkel significant bij T12. Voor MRSA was de bacteriële belasting bij NDT-PVA significant lager in vergelijking met de andere behandelingen vanaf T6. Er werd geconcludeerd dat NDT-PVA de grootste reductie in bacteriële belasting gaf, maar deze dressing moet wel *in vivo* getest worden om zijn invloed op de wondheling te gaan.

In **hoofdstuk 6** werd de meerwaarde van NDT voor per secundam heling van wonden aan het onderste lidmaat bij paarden nagegaan, gebruik makende van de technieken beschreven in de vorige hoofdstukken. De meerwaarde van NDT werd eerst getest in een acuut niet-geïnoculeerd wond model (hoofdstuk 6.1) en daarna in een geïnoculeerd wond model (hoofdstuk 6.2).

In **hoofdstuk 6.1** werd de meerwaarde van NDT op de ‘per secundam heling’ van acute wonden aan het onderste lidmaat van paarden nagegaan in vergelijking met Ca-alginaat dressings. Twee circulaire wonden van 3,5 cm diameter werden gecreëerd op beide metacarpi van 5 paarden. Tijdens de eerste 9 dagen werd één lidmaat behandeld met NDT, terwijl het contralaterale lidmaat behandeld werd met Ca-alginaat dressings (referentie). Vanaf dag 9 tot 71 werden beide ledematen onder verband gehouden met hydrofiele

polyurethaanschuim dressings. Tijdens het helingsproces werden de wond dimensies gemeten, swabs genomen voor kwantitatieve bacteriologie en werd de doorbloeding indirect gemeten met een thermografische camera. Op 5 tijdstippen werd een 8 mm punch biopt genomen voor zowel de analyse van groeifactoren, histologisch scoren van ontsteking en herstel, als voor het immunohistochemisch aantonen van B-cellen, T-cellen, macrofagen, endotheel, en myofibroblasten. Het wondoppervlak van NDT-wonden was significant kleiner in vergelijking met referentie wonden tijdens de 9 dagen durende behandelingsperiode (P = 0,005) en in de vroege periode na de behandeling (P = 0,003), maar niet in de late periode na behandeling. NDT-wonden waren significant dieper in vergelijking met referentie wonden in de behandelingsperiode (P = 0,007) en in de vroege periode na behandeling (P = 0,029), maar niet in de late periode na behandeling. Referentie wonden vertoonden een significant hogere score voor acute ontsteking (P = 0,044) in vergelijking met NDT-wonden. In de NDT-wonden weren er daarentegen significant (P = 0,008) meer macrofagen aanwezig. Er was geen significant verschil in de histologische herstelscores en concentraties van groeifactoren, noch in de aanwezigheid van B-cellen, T-cellen, myofibroblasten en neovascularisatie. Op basis van deze resultaten kon geen meerwaarde worden gedetecteerd voor het gebruik van NDT in plaats van Ca-alginaat dressings voor de behandeling van acute ‘per secundam’ helende wonden aan het onderste lidmaat van het paard.

In **hoofdstuk 6.2** werd de meerwaarde van NDT onderzocht voor de behandeling van gecontamineerde per secundam helende wonden aan het onderste lidmaat van paarden in vergelijking met zilver Ca-alginaat dressings. Twee circulaire wonden van 3,5 cm diameter werden gecreëerd op beide metacarpi van 5 paarden. Elke wonde werd geïnoclueerd met 2 veel voorkomende wondpathogenen bij het paard: *Staphylococcus aureus* en *Pseudomonas aeruginosa.* Tijdens de eerste 6 dagen werd één lidmaat behandeld met NDT, terwijl het contralaterale lidmaat behandeld werd met zilver Ca-alginaat dressings (referentie). Vanaf dag 6 tot 71 werden beide ledematen onder verband gehouden met hydrofiele polyurethaanschuim dressings. Tijdens het helingsproces werden de wond dimensies gemeten, swabs genomen voor kwantitatieve bacteriologie en werd de doorbloeding indirect gemeten met een thermografische camera. Op 5 tijdstippen werd een 8 mm punch biopt genomen voor zowel de analyse van groeifactoren, histologisch scoren van ontsteking en herstel, als voor het immunohistochemisch aantonen van B-cellen, T-cellen, macrofagen, endotheel, en myofibroblasten. Er kon geen significant verschil worden waargenomen tussen de behandelingen voor geen enkele van de wonddimensies (wondoppervlak, maximale diepte en volume). Er was ook geen significant verschil in bacteriële belasting, wondtemperatuur (dus doorbloeding) en zwelling van het lidmaat tussen NDT en referentie wonden. Bovendien waren er ook geen significante verschillen in de histologische scores

voor acute ontsteking en herstel, noch in de aanwezigheid van B-cellen, T-cellen, macrofagen, myofibroblasten en neovascularisatie. De concentraties van de groeifactoren transforming growth factor β1 en β3 was niet significant verschillend tussen de twee behandelingen. Gebaseerd op deze resultaten kon er geen meerwaarde worden vastgesteld voor het gebruik van NDT in plaats van zilver Ca-alginaat dressings voor de behandeling van gecontamineerde ‘per secundam’ helende wonden aan het onderste lidmaat van het paard.

Hoofdstuk 7 kadert de bevindingen van deze doctoraatsthesis in een bredere context, beschrijft de voornaamste conclusies en kijkt naar de toekomstige onderzoeksmogelijkheden. Deze doctoraatsthesis verschaft inzicht in de verschillende effecten van NDT op een acuut niet-geïnoculeerd wond model en een geïnoculeerd wond model bij het paard. In de toekomst zou het potentieel van NDT voor het verbeteren van huidtransplantaties bij het paard moeten onderzocht worden. Bovendien kan het gebruik van andere primaire dressings en studieontwerpen onze kennis over het effect van NDT op ‘per secundam’ helende wonden bij paarden uitbreiden. Ten slotte moeten wond modellen nog steeds verder worden verfijnd, alsook de methoden die gebruikt worden om wond helingsparameters op te volgen.

Lore Van Hecke werd geboren op 29 februari 1988 te Gent. Na het behalen van haar diploma secundair onderwijs aan het Sint-Franciscus Instituut te Melle (Wetenschappen-Wiskunde), begon ze in 2006 met de studie Diergeneeskunde aan de Universiteit Gent. In het jaar 2012 studeerde ze af als Master in Veterinary Medicine in de diergeneeskunde-afstudeerrichting paard met de grootste onderscheiding.

Gedreven door haar passie voor toegepast onderzoek, begon ze direct na haar studies met doctoreren aan de vakgroep Heelkunde en Anesthesie van de Huisdieren gefinancierd door het Bijzonder Onderzoeksfonds onder leiding van haar promotoren prof. Ann Martens en prof. Katleen Hermans. Tijdens dit doctoraatsproject voerde ze onderzoek naar het gebruik van negatieve druktherapie voor de behandeling van open wonden bij paarden.

Lore is auteur en coauteur van verschillende wetenschappelijke publicaties in peer-reviewed internationale tijdschriften. Ze gaf presentaties op verschillende internationale congressen, begeleidde studenten tijdens hun masterproef en vervolmaakte de doctoraatsopleiding van de Doctoral School of Life Science and Medicine aan de Universiteit Gent in 2017.

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“If we knew what it was we were doing, it would not be called research, would it?”

Albert Einstein