



Università degli Studi di Milano

**GRADUATE SCHOOL OF VETERINARY SCIENCES
FOR ANIMAL HEALTH AND FOOD SAFETY**

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Doctoral Program in Veterinary Clinical Sciences

XXVIII CYCLE

Academic Year: 2014-2015

**APPLICATION OF HYALURONIC
ACID IN THE HEALING OF NON-
EXPERIMENTAL OPEN WOUNDS:
A PILOT STUDY ON 12 WOUNDS
IN 10 CLIENT-OWNED DOGS**

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CHAPTER 1

Introduction

1. Introduction

Veterinarians have frequently to deal with wounds to the skin, subcutis, and underlying muscles and in many cases, a second intention healing with contraction and epithelialization is required.¹⁻³ The management of the wound varies according to wound stage and generally includes irrigation, mechanical, and chemical debridement, adherent and non-adherent dressing and a range of topical medication.⁴ Recently, many veterinary studies have been focused on wound care, especially on new healing strategy, techniques, and products such as the use of advance medical dressing and negative pressure therapy with the goal to accelerate the healing process.⁵⁻¹²

The present study would like to better elucidate the possibility of using Acid Hyaluronic-based medications for accelerating the healing process in spontaneous open wounds in dog.

1.1 The healing of the skin

Wound healing is a dynamic process composed by the sequence of different phases: Inflammation, Proliferation (also known as repair), and Maturation – that overlap in time.¹

- Inflammation

The blood clot generated after disruption of blood vessels and extravasation of blood constituents caused during a tissue injury, not only re-establishes haemostasis but also provides a provisional extracellular matrix for cell migration.¹³ The release of vasoactive mediators and chemotactic factors from platelets and injured parenchymal cells recruits inflammatory leukocytes to the site of injury. The wound area starts to be clean from foreign particles and bacteria by infiltrating neutrophils.¹³ At the same time, macrophages bind to specific proteins of the extracellular matrix by their integrin receptors stimulating phagocytosis of microorganism.¹⁴ In addition the presence of monocyte- and macrophage-derived growth factors are almost certainly linked to the initiation and propagation of new tissue formation in wounds making possible the hypothesis that macrophages have a pivotal role in the transition between inflammation and repair.¹⁵⁻¹⁶ During this phases the classic sign of inflammation such as redness, swelling, heat and pain are well detectable and are the result of vasodilatation, fluid escape and obstruction of the local lymphatic vessels.¹

- Proliferation (Repair or remodelling phase)

During this phase proliferative processes such as angiogenesis, fibroplasia and epithelialization occur. The transition from the inflammatory to the proliferation phase is marked by the invasion of fibroblast that leave collagen on the wound

bed, and by the migration and formation of new endothelial structures. The combination of these cells forms the red fleshy granulation tissue that fill the wound bed, characterizing this healing phase.¹⁷ Re-epithelialization begins within hours after injury with epidermal cells at the margin of the wound that undergo phenotypic alterations that permit cells movement.^{13,17} As re-epithelialization occurs, a progressive accumulation of new basement membrane material under the migrating cells is deposited, starting from the margins and continuing inward. Over time, the epidermal cells revert to their normal phenotype and become firmly attached to the basal membrane and underlying dermis.¹⁷ During the repair phase, another process called “contraction” occurs. Wound contraction involves a complex and superbly orchestrated interaction of cells, extracellular matrix, and cytokines.^{13,17} In particular the orchestra is guided by a unique population of mesenchymal cells called myofibroblasts. These myofibroblasts contain varying amount of actin, vimentin and desmin that give to the cells contractile ability. During this process the wound have a characteristics stellate appearance due to the stretching of the surrounding skin. Contraction will continue until the wound edges meet or if the tension of the surrounding skin equals or exceed the force of contraction.¹⁷

- Maturation

Even in presence of complete re-epithelialization, a remodeling and reorganization of the connective tissue occur and may takes months, even years leading to a scar formation. This remodeling is characterized by a reduction in collagen content by decreased production and increased degradation. The growth in mechanical strength is extremely low and a scar achieve a maximum strength of only 70-80% of the original tissue.¹⁷

1.2 The management of open wound in Veterinary Medicine

Despite in nature many open wounds in wild animals heal without the care of a veterinarian; in more serious injuries the delays in early effective management could have devastating consequences. The clinician can reduce the likelihood of complications and pain while improving the chances of a satisfactory functional and cosmetic outcome.¹

The clinician has to understand the normal process of healing and what the timeframe should be for normal tissue to achieve granulation, control of sepsis and epithelialization.¹⁷ Consequently the management of the wound varies according to wound stage with the aim to optimize the conditions for wound healing.^{3,4}

Pavletic in his book described six basic steps to approach the management of open wounds¹:

1. Prevention of further wound contamination;
2. Debridement of dead and dying tissue;
3. Removal of foreign debris and contamination;
4. Provision of adequate wound drainage;
5. Promotion of a viable vascular bed;
6. Selection of the appropriate method of closure.

To answer to these 6 steps veterinarian generally performed irrigation, mechanical, and chemical debridement, and cover the wound with adherent and non-adherent dressing and a range of topical medication.⁴

1.2.1 Advanced medical dressing in Veterinary Medicine

In veterinary medicine, many recent studies have been focused on new healing strategy and products with the aim to accelerate the healing rate. In this scenario, experimental and clinical studies were performed in animals using advanced medical dressing already listed in the management of human open wounds.⁵⁻¹²

Some of them have listed below:

- *Honey*

The use of honey for human open wounds was already tested 3000 years ago, but its role have been just recently re-evaluated from the modern medical world.¹⁸ Its low Ph, the amount of H₂O₂ and its osmolality give to honey an antimicrobial activity and seem to participate to the control of inflammatory cytokines release, fibroblast and vascular deposition.¹⁹ Many different honey-based dressing are available. In veterinary medicine the use of manuka honey was tested on experimental open wound in horses. At same time point, the daily application of manuka honey led to a decrease in wound size respect to non-treated wound.⁸

- *Maggot-therapy*

Maggot-therapy involves the use of disinfected fly larvae directly applied on the wound bed. Those larvae have the ability to debride the wound from necrotic tissue, reduce bacterial contamination due to the antimicrobial activity of their secretory products and enhance the formation of healthy granulation tissue.^{20,21} In human medicine, the application of maggot-therapy is reserved as the last line treatment of chronic wounds unsuccessfully treated after a long period with others advance medical strategy.²¹ In veterinary medicine the experience about

the use of maggot to debride wounds is limited to few case reports and small cases-series.²²⁻²⁵ Many, in fact, are the possible disadvantages of this therapy such as pain, difficult to built a correct dressing, potential iatrogenic ammonia toxicity and excessive bleeding if wound is close to major vessels.²⁵

- *Aloe Vera*

Aloe Vera is a plant well-known for its anti-inflammatory, anti-oxidative, anti-microbial and immune system modulation properties. Although its large use in human dermatology, experimental studies focused on its application for accelerate the healing of open wound led to contradictory results.²⁶ In veterinary medicine two studies were published but the results are still inconsistent to suggest the application of Aloe Vera in infected open wounds in animals.²⁷⁻²⁸

- *Platelet-rich plasma and platelet gel*

The platelet gel (PG) is defined by the Italian Board of Health as autologous or heterologous hemocomponent obtained by activation of hyper-concentrated platelets pellets with calcium and others activating substances (Ministerial Decree March 3rd, 2005).²⁹ In human medicine the application of platelet-rich plasma (PRP) was performed successfully in many disorders, from ophthalmology to orthopaedics, from oromaxillofacial surgery to cutaneous ulcers.³⁰⁻³⁴ As in human, the interest on clinical application of PG growths also in veterinary medicine and many clinical experiences were published confirming the positive impressions on healing rate.^{12,35,36} In particular, the randomized controlled clinical trials of Tambella et al. (2014) proved the efficacy of PG on chronic, bilateral, decubital ulcers in dogs.¹² The

exact mechanism of action behind these results is not fully understood. An hypothesis is based on the fact that platelet releasing numerous growth factors (GFs), plays an important role in the remodelling phase of healing so the use of this PG and PRP imply an increase of GFs on the wound bed. More recently the good result reported in chronic wound has been also confirmed in small experimental acute wound in beagle dogs.³⁷ As many studies have confirmed the advantages in use PRP and PG in many veterinary disorders, as also commercially available devices to obtain this products have been released. The study of Franklin et al (2015) tests five different commercial systems and finds out that the characteristics of the PRP products differed considerably, suggesting that clinicians should be aware of the data, or lack of them, supporting use of a particular PRP for a specific medical disorder.³⁸

- *Mesenchymal stem cell*

Stem cells are capable to differentiate into several tissues of the body and because of this ability they have been used for the healing of acute and chronic wounds. They can originate from various sources such as bone marrow, peripheral blood, and umbilical cord.³⁹⁻⁴² Autologous or allogenic mesenchymal stem cells (MSC) derived from bone marrow have demonstrated to enhance wound healing, increase blood vessel and granular tissue formation. Taking into consideration the donor site morbidity and pain for harvesting bone marrow, in human medicine another source such as adipose tissue has been investigated.⁴³

In veterinary medicine one study reported the use of allogenic MSCs from bone marrow on a canine cutaneous wound model. MSC-treated wound showed a more rapid healing rate than control wounds, and

this was hypothesized to be linked to the MCSs promoting re-epithelialization.⁴⁴ Another case-report reported the combined use of PRP and adipose-derived MSC to improve the healing rate on a large traumatic cutaneous wound in a dog.⁴⁵

- *Negative Pressure Wound Therapy*

This healing strategy involves the use of a device that induce the presence of localized negative pressure on the wound surface to promote wound contraction, angiogenesis and removal of excess fluid. This treatment technique was developed in the 1990s for the management of chronic wounds but nowadays has been recommended for virtually all kinds of acute and chronic wounds in human medicine.⁴⁶ Since 2005 in veterinary literature case report have been published on the use of negative pressure wound therapy (NPWT) in horses, dogs, cats and tortoise.⁴⁷⁻⁵¹ More recently case-control experimental studies and large case-series clinical studies in dogs have been published.^{5,11,52,53} The NPWT seems accelerate the deposition of a smooth, red and healthy granulation tissue optimizing the wound for bed surgical closure or second intention healing.^{5,11} At the same time epithelialization seems to be retarded suggesting that little benefits can be achieved in continuing NPWT for long period (over 10 days).¹¹ Using NPWT the bandage can be change every 2-3 days but one of the problem encountered is the maintenance of a sub-atmospheric pressure under the dressing. Pitt and colleagues (2014) described only minor complications in dogs that decreased in frequency while staff gained experience in applying dressings and became familiar with the machines. Even if some home management experiences, the use of

NPWT in veterinary medicine is generally reserved to hospitalized patients.⁵

- *Hyaluronic Acid*

Hyaluronic acid (HA) is a glycosaminoglycan consisted of a basic unit of two sugars, glucuronic and N-acetyl-glucosamine, that forms a part of the extracellular matrix.⁵⁴ Many *in-vitro* and *in-vivo* studies confirmed that HA is closely involved in fibroblast proliferation, enhanced formation of granulation tissue and angiogenesis, and even in keratinocyte proliferation and migration during wound healing process.⁵⁵⁻⁶⁰ These promising results associate with a non-immunogenic response and its degradation by natural, non-inflammatory clearance mechanisms have led to the development of a range of wound dressing containing HA for human medical use.^{55,61-65} In human medicine the role of HA in improving the healing rates, no matter the form in which is delivered (gel, film, sponge) is well known for wound ulcers of various etiologies, burns and epithelial surgical wounds.⁶⁴ In veterinary medicine, the information about the use of HA as a part of wound management is little, and nowadays, the benefit results of this molecule application on wound healing as a cross-linked HA-based dressing in experimental dog court are still controversial.^{6,7} The first study was published in 2011.⁶ In this study including cross-linked thiolated carboxymethylhyaluronic acid (CMHA-S) based gel and film were applied on experimental wounds in rats, dogs and horses. Even if in dogs the treated wounds tended to be smaller than control group, only in horses this tendency became statistically evident. The gross appearance of the wounds in both species indicated a healthier granulation tissue and more epithelialization in treated wounds than

control.⁶ The second study published in 2013 included only beagle dogs in which experimental 2x2 cm wounds on the trunk were treated with the CMHA-S-based gel.⁷ Although the hypothesis that CMHA-S accelerated the healing rate was rejected, by the end of the study CMHA-S-treated wound seems to contract more and, by a subjective evaluation, results in a smoother granulation tissue and a less-obvious scar respect to control wound.⁷ These results suggested that in veterinary patients additional investigations should be advice to better elucidate the effects of HA on open wounds' healing.

1.3 History of the hyaluronic acid in wound management

Karl Meyer and John Palmer in 1934 described a procedure for obtaining of a novel glycosaminoglycan from the vitreous humour of bovine eyes.⁶⁶ This substance contained an uronic acid and an aminosugar so was named “hyaluronic acid”, and it’s sometimes referred as “hyaluronan” due to the fact that it exists in vivo as a polyanion and not in the protonated acid form. Subsequently in 1964 this molecule was synthesized in vitro and few years later, in 1968, was therapeutically used for the first time on a burn.⁶⁷⁻⁶⁸

Since its discovery, several studies were focused on the role of HA in the organisms. Hyaluronic acid is one of the largest components of the extracellular matrix (ECM) and it is conserved throughout all mammals. It is found in high concentrations in several soft connective tissue, including skin.⁵⁵ In fact, HA plays a vital role in maintaining tissue integrity, as well as in facilitating adhesion and differentiation of cells during inflammation, wound repair, and embryonic development.^{56,57} Particularly in the early stage of wound repair, HA provides a temporary structure that facilitates the diffusion of nutritional supplies and helps rid the wounds of waste products from cell metabolism.⁶⁹ In addition, HA is subsequently closely involved in keratinocyte proliferation and migration.⁵⁸

In the physiological condition, HA is highly hydrophilic, surrounded by a sphere of water molecules linked by hydrogen bonds. This role of hydration underlines the central role of HA in the skin. Otherwise, the water solubility, the rapid resorption and short residence time in tissue of the purified form of HA limited its possible therapeutic application. Taking into consideration these aspects, several attempts have been made to modify its molecular structure to obtain a more stable solid material without delete the inert and non-inflammatory properties of HA.⁷⁰ This led between 80’s and 90’s to the development of

hyaluronan esters from the esterification of free carboxyl groups of glucuronic acid present along the molecular chain back-bone.^{71,72}

Alcohol esterification, generally benzyl alcohol, prevents water ingress and it is the usual method of stabilisation. In essence, this cross-links the polymer to a variable degree leading to molecules with decrease solubilities. Additionally, esterification prevents fibroblast binding, so reducing cellular degradation: the 75% esterified materials is degraded over 7-14 days, whilst the 95% of the product could be present in tissue sections after up to 2 months.^{70,73}

The improvement of tissue-engineering techniques in human medicine has taken to a new application of HA-based materials that have not only been applied in the traditional way where a degradable material may be used on its own, but also as a support matrix or as a substrate for the delivery of cultured cells or for three-dimensional tissue reconstruction.^{70,74-76}

1.4 Wound bed preparation and TIME principles in human medicine

One of the major problems in human medicine is to face with chronic wounds. Some wounds do not follow a linear progression through the well-known phase of healing and in some point of the process abnormality can occur leading to a non-healing wound.⁷⁷

Wound bed preparation (WBP) attempted to correct chronic wound management with the initial aim of preparing the wound to accept advanced wound healing therapies. In fact, it became clear that an advance therapy applied on a poorly prepared wound bed could not succeed. The preparation of the bed included attention to viable tissue, control of bacterial burden, control of inflammation and moisture balance, and stimulation of the tissue migration from the wound edges.⁷⁷

From the initial aim, WBP soon evolved into a method that could be used to stimulate the endogenous process of wound repair, even without the need of advanced therapies. In 2000, Sibbald et al. defined WBP as a “changing paradigm that links treatment to the cause and focuses on three components of local wound care: debridement, wound-friendly moist interactive dressings and bacterial balance”.⁷⁸

These three components are a toll for systematically assessing the wound in terms of tissue, infection/inflammation and moisture and for carrying out interventions based on and understanding of the underlying cellular disruption. Otherwise the last aspect of a wound was not considered. In fact, the ultimate aim was to close the wound by stimulating the migration of epidermal tissue from the wound edges.⁷⁷

Adding this fourth component, in 2002 the acronym TIME was released:⁷⁹

- T for tissue: non viable or deficient
- I for infection/inflammation
- M for moisture balance
- E for edges of wound

Wound bed preparation became a cyclical process of wound management where assessment was followed by intervention, which in turn was followed by further assessment.⁷⁷

Although WBP and TIME have focused on correcting abnormalities inside pre-existing chronic wounds, potentially all acute wounds may become chronic if recovery is not optimal, so even acute wounds could benefit from WBS and TIME concepts with the aim to not translate them into non-healing wounds.⁷⁷

1.5 The assessment of healing

One of the problems encountered in the evaluation of the healing process is also how to evaluate it. The high number of assessment methodologies published in human medicine reflects the complexity of objective wound healing assessment and the ongoing need for an ideal method.⁸⁰ Despite no methods was find adequate for all wound types,⁸⁰ appropriate and periodic wound evaluation has been shown to be a moderately sensitive and specific way to alert clinicians to a less than optimal treatment outcome or a need of re-evaluation of the therapeutic strategy in that patient.^{81,82} In addition monitoring the wound healing can help the communications among clinicians by defining a common language and standardising assessment of wound characteristics, at least in the same medical institution.⁸³

The end point of the healing process is to obtain the *restitutio ad integrum* of the skin layer, and the progressive reduction of wound area is the mainstay parameter to assess. Despite this consideration, a single characteristic can neither provide all the necessary data to determine the adequacy of the treatment plan, nor allow the progress and the degeneration of the wound.⁸⁴ In human medicine the dimensional assessment is associated with visual and physiological evaluations.⁸¹ Scale scoring systems, particularly for chronic wounds, were available in human medicine. Points are given according to many parameters and than a total score is calculated.^{80,82,85} Wound that shows a plateau in the total score in 2 to 4 weeks are generally considered non-progressive and it should be reevaluated to determine why healing has stopped and if treatment change is necessary.⁸²

In veterinary studies the dimension of the wound surface is the sole parameter evaluated to determine the healing progression but recently the addition of other

factors such as characteristics of the wound bed and of the periwound tissue have been discussed, but not yet evaluated, as other quality parameters for verifying the healing process and also the wound care therapy application.^{7,11}

CHAPTER 2

Aim of the study

2. Aim

Due to the little information on literature, the aim of this study was to collate a sample of canine patient with full-thickness skin wounds healed by second intention and to explore on theme the use of available in commerce HA-containing wound dressing, detailing the difficulties, adverse events, costs, and wound outcome. In addition to wound dimensions, the progresses of the wounds were also followed using two wounds assessment scales borrowed from the human medicine that quantitatively described the status of salient physiologic wound characteristics.

CHAPTER 3

Materials and Methods

3. Materials and Methods

3.1 Ethical approval

This study was performed at the Department of Clinical Science and Public Health of the University of Milan. It did not include experimental animals and it was performed on client-owned dogs referred to our institution for therapeutic purpose. All owners gave us the consensus for treatments, measurements and for data recording. All clinical procedures were performed in accordance with Italian law (DL 14th march 2014 n.26) and Europe Union legislation covering the use of animals for scientific purpose (“Animal Scientific Procedures Act” 63/2010/EU).

3.2 Inclusion Criteria

Animals prospectively included in the study were client-owned dogs with cutaneous open wounds healed completely by only second intention consecutively referred from January 2013 to February 2014. All wounds had at least a full-thickness skin lost and were treated using available in commerce HA-containing wound dressing from the date of admission to the date of complete re-epithelialization. Due to the absence of specific veterinary formulation in our country, HA-based medications available in commerce for human medicine were used, and informed owner consent was obtained for each dog. The complete re-epithelialization was assessed independently by two veterinary surgeons. All animals included had to be followed-up at least until complete re-epithelialization of the wound. Any wound in which was achieved a primary, a delayed primary or a secondary closure were excluded from the study.

All dogs included underwent a physical examination before starting any evaluation and treatment of the wound. For patients referred for traumatic

injuries any possible reason of life-threatening were excluded or managed before starting cutaneous open wound evaluation.

Other data recorded in the clinical record were: Signalment, weight and body condition score (BCS - scoring from 1 to 5)⁸⁶, etiology (if known), site of the lesion, complete blood sample evaluation (complete blood count, biochemistry), any concomitant drugs administration (e.g. corticosteroids, anti-blastic therapy), and any concomitant disease (e.g. bone fracture, cavitary collections, metabolic, or endocrinologic disease). If referring veterinarian had already treated a wound before referred it, type of treatment and timing of treatment were recorded.

3.3 Wound Assessment Methods

The wound area was calculated using two methods. First, each wound has been approximated to a rectangular figure, and the area was calculated multiply the two maximum perpendicular diameters measured in a bi-dimensional representation. The two measures were achieved using a digital caliper or in presence of wound above the curves of the body whit a soft meter and the limits for the evaluation of the diameters were the macroscopic margins of epithelial tissue. Secondary, a digital photograph of the wound was obtained at each clinical control and uploaded in a wound tracing software program (NIH Image] software, <http://rsb.info.nih.gov/nih-image/>) that calculated the wound area. For both methods, wound area was interpreted as 100% on admission and the areas on subsequent controls were expressed as a percentage of admission value using the formula:

$$A_t/A_0*100$$

Where, A_0 is the area on admission and A_t is the wound area on control date.

All wounds included in the study were also prospectively classified at admission and during healing process by means of two scale scoring systems, Bates-Jensen

Wound Assessment Tool (BWAT) and wound bed scoring (WBS).^{85, 87-90} The BWAT includes 4 parameters scoring from 0 to 5 (size, depth, edges, undermining) and 9 parameters scoring from 1 to 5 (necrotic tissue - type and amount, exudate - type and amount, surrounding skin color, peripheral tissue - edema and induration, granulation tissue, epithelialization). The wound assessment results by adding the score of each parameter from a minimum of 9 (the best score) to a maximum of 65 (the worst score (Figure 1)).^{85,87-89} The WBS comprised an evaluation of 8 parameters (black eschar - eczema/dermatitis - depth - scarring - color of wound bed - edema/swelling - resurfacing epithelium - exudate amount) scoring from 0 to 2. Using this scale a wound could range from a maximum of 16 (the best score) to a minimum of 0 (the worst score) (Figure 2).⁹⁰ In both scales in the presence of multiple features of the same parameter the worst score was reported. Two surgeons independently performed the assessments. The quality and type of exudate were evaluated on the basis of how the removed bandage looked like while all other parameters were assessed after irrigation (with saline) by means of 20 ml syringe and a gentle sterile gauze sponges handling with the purpose to remove the superficial biofilm.

Figure. 1 Bates-Jensen Wound Assessment Tool (BWAT)

Item	Assessment
Size (length x width)	0. Healed, resolved wound 1. < 4 cm ² 2. 4-16 cm ² 3. 16.1 < 36 cm ² 4. 36.1 < 80 cm ² 5. > 80 cm ²
Depth	0. Healed, resolved wound 1. Non-blanchable erythema on intact skin 2. Partial thickness skin loss involving epidermis and/or dermis 3. Full thickness skin loss involving damage or necrosis of subcutaneous tissue; may extend down to but not through underlying fascia; and/or mixed partial and full thickness and or tissue layers obscured by granulation tissue 4. tissue layers obscured by necrosis 5. Full thickness skin loss with extensive destruction, tissue necrosis or damage to muscle, bone or supporting structures
Edges	0. Healed, resolved wound 1. Indistinct, diffuse, none clearly visible 2. Distinct, outline clearly visible, attached, even with wound base 3. Well-defines, not attached to wound base 4. Well-defined, not attached to base, rolled under, thickened 5. Well-defines, fibrotic, scarred or hyperkeratotic
Undermining	0. Healed, resolved wound 1. None present 2. < 2 cm in any area 3. 2-4 cm involving < 50% wound margins 4. 2-4 cm involving > 50% wound area 5. > 4 cm or tunnelling in any area
5. Necrotic tissue type	1. None visible 2. White/grey non viable tissue and/or non-adherent yellow slough 3. Loosely adherent yellow slough 4. Adherent, soft, black eschar 5. Firmly adherent, hard, black eschar
6. Necrotic tissue amount	1. None visible 2. <25% of wound bed covered 3. >25-50% of wound bed covered 4. >50-75% of wound bed covered 5. >75-100% of wound bed covered

7. Exudate type	<ol style="list-style-type: none"> 1. None 2. Bloody 3. Serosanguineous: thin, watery, pale red/pink 4. Serous: thin, watery, clear 5. Purulent: thin or thick, opaque, tan/yellow, with or without odor
8. Exudate amount	<ol style="list-style-type: none"> 1. None, dry wound 2. Scant, wound moist but no observable exudate 3. Small 4. Moderate 5. Large
9. Skin colour surrounding wound	<ol style="list-style-type: none"> 1. Pink or normal for ethnic group 2. Bright red and/or blanches to touch 3. White or grey pallor or hypopigmented 4. Dark red or purple and/or non-blanchable 5. Black or hyperpigmented
10. Peripheral tissue edema	<ol style="list-style-type: none"> 1. No swelling or edema 2. Non-pitting edema extends < 4 cm around wound 3. Non-pitting edema extends > 4 cm around wound 4. Pitting edema extends < 4 cm around wounds 5. Crepitus and/or pitting edema extends > 4 cm around wound
11. Peripheral tissue induration	<ol style="list-style-type: none"> 1. None present 2. Induration, < 2 cm around wound 3. Induration 2-4 cm extending < 50% around wound 4. Induration 2-4 cm extending > 50% around wound 5. Induration > 4 cm in any area around wound
12. Granulation tissue	<ol style="list-style-type: none"> 1. Skin intact or partial thickness wound 2. Bright, beefy red; 75 to 100% of wound filled and/or tissue overgrowth 3. Bright, beefy red; < 75 to >25% of wound filled 4. Pink and/or dull, dusky red and/or fills 25% or less of wound 5. No granulation tissue present
13. Epithelialization	<ol style="list-style-type: none"> 1. 100% wound covered, surface intact 2. 75-100% wound covered and/or epithelial tissue extends >0.5 cm into wound bed 3. 50-75% wound covered and/or epithelial tissue extends to < 0.5 cm into wound bed 4. 25-50% wound covered 5. <25% wound covered

Figure 2. Wound bed scoring (WBS)

Wound Bed Score			
	Scores of 0	Scores of 1	Scores of 2
Black Eschar	0	1	2
Eczema/Dermatitis	0	1	2
Depth	0	1	2
Scarring (fibrosis/callus)	0	1	2
Color of wound bed	0	1	2
Oedema/Swelling	0	1	2
Resurfacing epithelium	0	1	2
Exudate Amount	0	1	2
Add scores for each column →			
TOTAL SCORE			

3.4 Wound management protocol

Because wound healing is a dynamic process, all wounds were prospectively managed with HA-containing dressing based on TIME principles [35] in accordance to the characteristics of the open wound and also to manufacture's instruction. In presence of necrotic tissue (BWAT amount of necrotic tissue score ≥ 2 and/or WBS black Eschar score ≤ 1) a debridement (surgical sharp debridement [SD] and/or medical debridement [MD]) were always applied. The MD consisted of HA plus collagenase-based topical enzymatic debridement agent (Bionect Start®, Fidia Farmaceutici S.p.A., Abano Terme - PD, Italy) or a HA plus sodium alginate sterile micro-granules medication (Hyalogran®, Fidia Farmaceutici S.p.A., Abano Terme - PD, Italy). In absence of macroscopic necrotic tissue (BWAT amount of necrotic tissue score = 1 and WBS black eschar score = 0) an adsorbent medication made of partial benzyl ester derivative of hyaluronan (Hyalofill-F®, Fidia Farmaceutici S.p.A., Abano Terme - PD, Italy) was used when granulation tissue was forming or, when wound did not include deep tissue damage, a daily application of a spray composed by HA plus silver (Hyalosilver®, Fidia Farmaceutici S.p.A., Abano Terme - PD, Italy) was applied. When the open wound was entirely filled by granulation tissue (BWAT granulation tissue score = 2 and WBS color of wound bed = 2) the owners were instructed to daily apply a HA based topical medication (Connettivina®, Fidia Farmaceutici S.p.A., Abano Terme - PD, Italy) until complete epithelialization. If different and simultaneous features characterized different part of the wound a mix of this product was used. In all cases, the second layer of the bandage was composed by cotton padding and then a third layer of the latex-coated cohesive bandage (Easifix Cohesive®, BSN medical, Vibraye, France) was applied. A tie-over bandages was used to fix the second layer in area difficult to cover (e.g. inguinal area).

First wound area and assessments evaluation were performed the date of the first clinical examination for traumatic and primary closure complication wounds (CW), or the date of surgery for excision wounds. After, the assessments and wound area measurement were repeated at every bandage change performed by clinicians. All the bandage changes were sterilely performed. If needed or require by the owner adjunctive control respect to the clinical examination scheduled were performed, and the cause of earlier evaluation was recorded. Numbers and frequency of wound assessment for each animal were also reported. In the case of surgical SD all the assessments, including wound area measurements, were performed both before and after to evaluate the impact of the surgical procedure. When owners applied the HA-medications at home, a weekly clinical examination to assess the wound progress was scheduled. During each clinical examination, owners were asked to inform of any difficulties or disadvantage retrieved with the bandage. The presence of complete wound healing was assessed by the evaluation of the clinician and corresponded to a wound covered entirely by epithelial tissue in the absence of any sign of granulation tissue. Time of complete wound healing (TWH) was recorded and defines as the time from first assessment to complete re-epithelialization (100% of the wound area).

When complete re-epithelialization was assessed cosmetic and functional outcome were also evaluated and recorded in the clinical record.

During the study period, the use of antibiotic therapy was recorded. The use of antibiotics was reserved to wounds showed sign indicative of infection such as: Severe soft tissue swelling, erythema, pain, hyperthermia and/or purulent discharge. The antibiotics were chosen on the basis of anti-biogram results if a swab sample or a biopsy of the wound bed for microbiological culture was available. The antibiotic administration was stopped when the signs of infection were completely regressed, and a healthy granulation tissue (BWAT granulation tissue score = 2 and WBS color of wound bed = 2) was forming.

When the animals underwent general anesthesia, anesthetic protocol consisted of a multimodal approach including analgesic therapy. The protocol differed among animals, and it was calibrated on the basis of the clinical examination of the single animal and the type of procedure that had to be performed (surgical SD vs. bandage change). In all cases after surgical debridement and in every case in which pain was detected an analgesic therapy consisted of non-steroidal anti-inflammatory drug oral administration was prescribed to obtain pain relief.

3.5 Statistical Analysis

Pattern of wound area reduction (both methods) during follow-up was evaluated by analysis of variance model (ANOVA) for repeated measures. As a pre-fixed schedule for clinical examination was not planned and follow-up time was heterogeneous, 4 times which were common for all subjects were considered: 7, 14, 21, 28 days. To account for the correlation of the measures within the same subject, a variable identifying subject was considered in the ANOVA model as random effect, whereas days was included as fixed effect by dummy variables (mixed model ANOVA). To compare the pattern of the WBS and BWAT with the pattern of wound area reduction the first two scales were transformed as percentages of scoring as follows. For BWAT, the score at time 0 was considered as 100%. The maximum reduction for the patient was the difference between the initial score and the best achieved score (MR BWAT: BWAT at time 0-9). For each time, the difference between the initial score and observed score was calculated (difference BWAT). The percentage was then obtained as:

$$100-(\text{diff BWAT}/\text{MR BWAT})*100$$

For WBS, the score at time 0 was considered as 100%. The maximum increase for the patient was the difference between the best score achieved and the initial score (MI WBS: 16-WBS at time 0). For each time, the difference between the

observed score and the initial score was calculated (difference WBS). The percentage was then obtained as:

$$100-(\text{diff WBS}/\text{MI WBS})\times 100$$

The pattern of the transformed scales was then analyzed by mixed model ANOVA. The probability of reaching 0% (the best achieved score) during time for wound area reduction, BWAT, and WBS scores was estimated by Kaplan–Meier method. This method allowed to estimate the median time to complete wound area reduction (and the best achieved score of BWAT and WBS). Due to the low number of patients and preliminary aim of this study no formal statistical test on ANOVA were performed. Results are reported as model estimated reduction and 95% confidence intervals (CI). No adjustment was performed by covariate effects.

The agreement between the BWAT and WBS scoring independently attributed to the patients by two veterinary surgeons was evaluated by the intraclass correlation coefficient (ICC) based on two-way repeated ANOVA model with random effects.

As two methods were used to evaluate wound area (the approximation to a rectangular and software), an explorative comparison was performed on all the available wound measurements. No one of the two methods could be considered as gold standard thus the “exact” wound area was approximate by the average of the two calculated areas. The comparison was then examined by plotting the differences between the two areas measures against their average, as suggested in the Bland and Altman plot. Since the aim was only explorative no limits of agreement were calculated but, following a practical perspective, the percentages of measurements differences within defined ranges were reported.

CHAPTER 4

Results

4. Results

4.1 Population

Twelve open wounds in ten dogs were enrolled in the study. Breeds were as follows: Mixed-breed (2), Jack Russel Terrier (2), Dogue de Bordeaux (1), Irish Wolfhound (1), Dogo Argentino (1), Whippet (1), Pinscher (1) and American Pit Bull Terrier (1). Four dogs were females (1 neutered) and 6 were males (2 neutered). At admission, mean age was 7.9 years (range 1-15 years) and mean body weight was 23.1 kg (range 5-47 kg). Two dogs had a BCS of 2, in 5 dogs the BCS was 3 and in the last 2 dogs was 4.

At admission, three dogs reported comorbidities related to past history: Cardiopathy (2) and diabetes mellitus (1). Two dogs reported in the recent clinical history (2 weeks or less before admission) one or more surgical malignant tumor excision in location not associated to the wound: Perivascular wall tumor (1), mast cell tumor (1) and femoral osteosarcoma (1). One dog was surgically treated for a foreign body abscess of the mandibular region 2 weeks before admission.

The hematological parameters at admission were unremarkable in all but two dogs in which hypoalbuminemia (1) and high glucose level (1, the dog with diabetes mellitus) were found.

4.2. Wound data

A total of 12 wounds were included (2 dogs had 2 simultaneous and anatomically split wounds) and treated with HA-based medications until complete re-epithelialization. All wounds were summarized in Table-1. The wounds were classified on the basis of the etiology in: Traumatic wounds (TW, 7), primary closure complication wounds (CW, 2) or excised wounds (EW, 3) in

presence of skin defect intentionally leaved by surgeon. The specific nature of trauma was reported in Table-1. Two TW were referred in presence of unhealthy and deteriorating wound appearance after 10 days of previously treatments: Surgical apposition of traumatized cutaneous margins (Table-1) and naftalina granules and saline solution irrigation (Table-1). Regarding wounds developed after malignant tumor excision, the histologic margins were infiltrated in two cases (Table-1). No one wounds presented healthy granulation tissue at time to admission.

At admission, the median wound area using the approximation to a rectangular figure was 32 cm² (min 5.40, max 561). In all cases were debridement (SD and/or MD) was applied the wound areas had an average increase of 14.4 cm² (Table-2).

At admission, the median wound area using the software was 22 cm² (min 2.67, max 417.29). In all cases were debridement (SD and/or MD) was applied the wound areas had an average increase of 10.19 cm² (Table-2).

At admission, the median BWAT score was 32.5, while for WBS was 11. In the 7 wounds in which debridement had been applied, the median BWAT score improved from 38 to 32, corresponding to a median decrease from admission score of 6, while the median WBS score improved from 8 to 11, corresponding to a median increase from admission WBS score of 2 (Table-2).

All dogs were discharged at home the day of admission and returned to the clinic only for dressing changes and clinical controls. The median numbers of return to the clinic for bandage change (excluding the date of admission) was 5 times, (range, 4-14 times). When dressing was applied by the owner wounds were generally rechecked once a week (median, 7 days; range, 2-16 days). When the adsorbent medication made of partial benzyl ester derivative of hyaluronan was applied, the dressing was always revised by the clinician, and bandages were generally changed every 4 days (median, 4 days; range, 2-7 days). In the 2 widest

TW (Table-1 and Figures-10 and -11) due to the amount of exudate a silver-pad foam (Cellosorb Silver®, Laboratoires URGO, ChenO, Cedex, France) was applied over the HA-based medication before the second layer of the bandage until scoring for the amount of exudate achieved 2 out of 5 for BWAT and 2 out of 2 for WBS. Due to the location and the size of the wounds a tie-over bandage was applied in both cases.

In 5 wounds (3 dogs) antibiotic therapy was administered. In wounds 3, 4, 5 (Table-1) a swab test was performed due to the presence of purulent discharged and peripheral tissue erythema. In these cases *Klebsiella pneumoniae* was retrieved and an antibiotic protocol with marbofloxacin was started as anti-biogram results suggested. In wounds 6 and 7, the dog had been already under enrofloxacin administration before the admission, and the protocol was continued until appearance of granulation tissue due to the nature of the lesion even if no particular signs of infection were presented at that moment.

Excluding the SD that was always performed under general anesthesia, only for the management of wound 4 (Table-1) sedation was performed at every bandage change (every 3 days) until day 10 from admission. This was the widest wound and for clinical control the dorsal recumbency was necessary creating particular discomfort for the dog during this initial period. All other bandage changes in this dog and all bandage changes in the other patients were performed with wakeful dogs.

All the products were well tolerated by dogs, and no side effect was retrieved. Only one dog was prone to removing the bandage, and earlier returns to the clinic than scheduled were necessary also because the owner did not follow the suggestion to use Elizabethan collar (Table-1). Some owners complained the presence of an unpleasant odor of the bandage. This was correlated to high amount of exudate and breaks down of the HA-based products but not to infection as also assessed by the improvement of the wound status. When spray

formulation was used, owners reported that some dogs felt a little uncomfortable sensation due to the noise. The cosmetic result was considered good even if in all cases an alopecia area of different dimension has been retrieved. In addition, the skin in this area looks thinner and in some cases hyper-pigmented but no complication was reported related to these condition. No range motion alteration due to contracture were observed even for wounds located close to flank, axillar or other joint, and the functional outcome was judged great in all dogs.

4.3 Outcome

The median TWH was 34 days. The 0.25, 0.50, and 0.75 cumulative probability to be healed in this study was reached at 21, 34, and 54 days, respectively (Figure-3). No wounds were considered healed at 14 days, 3 were considered healed within 21 days (cumulative probability of healing: 0.25; 95% CI: 0-0.46), 5 wounds within 28 days (cumulative probability of healing: 0.42; 95% CI: 0.06-0.64), 7 wounds within 35 days (cumulative probability of healing 0.58; 95% CI: 0.19-0.79), and 10 wounds within 63 days (cumulative probability of healing 0.83; 95% CI: 0.41-0.95) (Table-1).

In all wounds the best BWAT score was achieved at the same date when clinician assessed the complete healing. Eleven out of 12 wounds achieved the minimum BWAT score of 9 while 1 wound achieved the score of 10. The pattern of cumulative probability to achieve the best BWAT score was superimposable to that previously reported for wound area reduction (Figure-3). Considering the WBS scale, the 0.25, 0.50, and 0.75 cumulative probability to achieve the best WBS score was reached at 9, 14, and 35 days, respectively (Figure-3). Two wounds reached the best WBS score within 7 days (cumulative probability 0.17; 95% CI: 0.00-0.35), 6 wounds reached the best WBS score within 14 days (cumulative probability 0.50; 95% CI: 0.12-0.72), 7 wounds

reached the best WBS score within 21 days (cumulative probability 0.58; 95% CI: 0.19-0.79) and no wound further reached the best WBS score between 21 and 28 days. In all cases, the best score was reconfirm in the subsequent assessments until complete epithelialization was achieved.

Considering the 1st month of treatment, the weekly mean percentages of two-axis wound area, software wound area, BWAT and WBS scores respect to admission values are illustrated in Figures 4-5-6-7, respectively.

When another clinician applied both scoring systems, the agreement between the two operators was considered good with an ICC of 0.88 for WBS (95% CI: 0.83-0.91) and of 0.95 for BWAT (95% CI: 0.93-0.97).

Regarding the comparison between area measurement with rectangular and software method, overall 107 measurements were performed with both the methods. Since 12 measurements for both method reported 0% at the time of complete healing, only the other 95 remaining measures were considered. As the differences between methods were related to the area, to facilitate the comparison data were subdivided according to the following categories: Areas <10 cm² (28.4% of the data), areas between 10 and 25 cm² (29.5% of the data), areas between 25 and 50 cm² (17.9% of the data), areas between 50 and 100 cm² (11.6% of the data) and areas beyond 100 cm² (12.6% of the data). Measurements with rectangular approximation tend to be greater than measurements with software and the differences between the two measurements tends to increase with the increasing of the area (Figure-8).

Table 1. Wounds included in the study

Case	Weight (kg) [BCS]	Age (years)	Type	Cause	Site	Area (cm ²)	TWH (days)	Concomitant drugs
1	38[4]	5	TW	laceration (unknown origin)	elbow	18	24	-
2	15[2]	1	TW	hot water pad burn	medial thigh	9.6	21	-
3	35[3]	4	TW	laceration (unknown origin)	distal forearm	36	62	-
4	21.7[4]	6	TW	car accident (degloving injuries)	ventral abdomen	540	75	-
5	21.7[4]	6	TW	car accident (shear injuries)	lomber region	38.7	35	-
6	9[4]	8	TW	electric pad burn	flank	109,6	54	-
7	9[4]	8	TW	electric pad burn	scapular	22,5	17	-
8	45[3]	4	EW	Malignant skin neoplasia excision	lateral hock	36	49	vinblastin + corticosteroid
9	5[3]	14	EW	Malignant skin neoplasia excision	proximal medial forearm	15.1	28	ACE-inhibitor + furosemide
10	6[3]	15	EW	Malignant skin neoplasia excision	caudal thigh	5,4	34	ACE-inhibitor + furosemide
11	47[3]	11	CW	Malignant skin neoplasia excision	lateral thorax	32	88	-
12	9.2[2]	11	CW	forelimb amputation for malignant skin neoplasia	shoulder	10.4	21	Insulin

Legend

TW: traumatic wound, EW= excisional wound, CW: primary closure complication wound.

Table 2. Wounds received debridement.

case	Type of debridement	Two-axis Area (cm ²) at admission	Two-axis Area (cm ²) post-debridement	Software Area (cm ²) at admission	Software Area (cm ²) Post-debridement	BW/AT at admission	BW/AT post-debridement	WBS at admission	WBS post-debridement
2	MD	9.6	19.2	2.67	8.74	33	28	7	12
3	SD	36	51.5	30.43	45.47	46	36	4	6
4	SD	540	561	401.65	417.29	55	45	3	9
5	MD	38.7	58.8	22.24	54.36	32	27	8	11
6	SD	109.6	139.5	4.8	5.43	38	32	11	12
7	SD	22.5	27.3	91.18	91.83	34	24	12	14
12	SD	10.4	11.2	17.83	18.99	38	34	10	11

Legend

MD=medical debridement, SD= surgical debridement. The assessment for wound underwent only medical debridement referred to day 4 when both the wounds achieved the widest area, for wound underwent surgical debridement referred to day 0 (when the surgery was performed).

Table 3. Comparison between “two-axis” and “software” methods for measuring the wound area.

	Two-axis Wound area < 9 cm ²	Two-axis Wound area > 9 cm ² and < 25 cm ²	Two-axis Wound area > 25 cm ² and < 50 cm ²	Two-axis Wound area > 50 cm ² and < 100 cm ²	Two-axis Wound area > 100 cm ²
Total number of measurements	24	31	17	11	12
Difference between the two methods					
< 1 cm ²	12 (50%)	5 (16%)	-	-	-
≥ 1 cm ² and < 2 cm ²	6 (25%)	1 (3%)	2 (11.8%)	1 (9.1%)	-
≥ 2 cm ² and < 3 cm ²	6 (25%)	3 (9.7%)	3 (17.6%)	-	-
≥ 3 cm ² and < 4 cm ²	-	2 (6.5%)	-	-	1 (8.4%)
≥ 4 cm ² and < 5 cm ²	-	4 (12.9%)	1 (5.9%)	2 (18.2%)	-
≥ 5 cm ²	-	16 (5.6%)	11 (64.7%)	8 (72.7%)	11 (91.6%)

Legend.

Difference greater than 1 cm² were found in 50%, 84%, and 100% of the areas within 10 cm², between 10 and 25 cm² and greater than 25 cm² respectively. Differences greater than 2 cm² were found in 25%, 81%, 88%, 91% and 100% of the areas within 10 cm², between 10 and 25 cm², between 50 and 100 cm² and greater than 100 cm², respectively. Differences greater than 5 cm² were found in 52%, 65%, 73% and 92% of the areas between 10 and 25 cm², between 25 and 50 cm², between 50 and 100 cm² and greater than 100 cm², respectively.

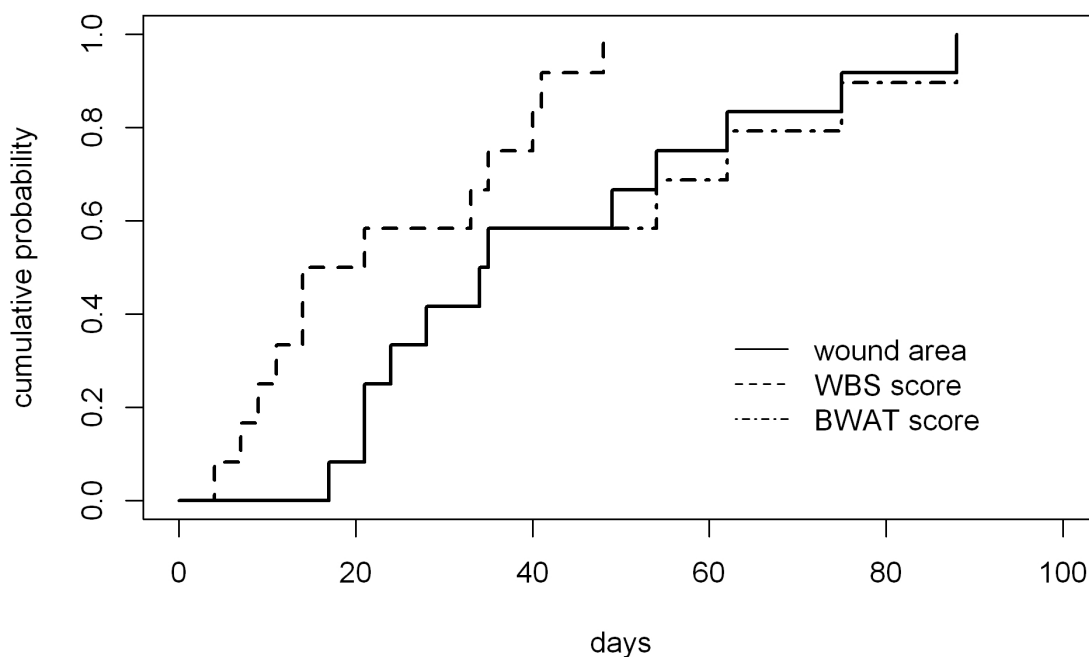


Figure 3. Cumulative probability to be healed considering complete wound area reduction and to reach the best BWAT and WBS scores during time.

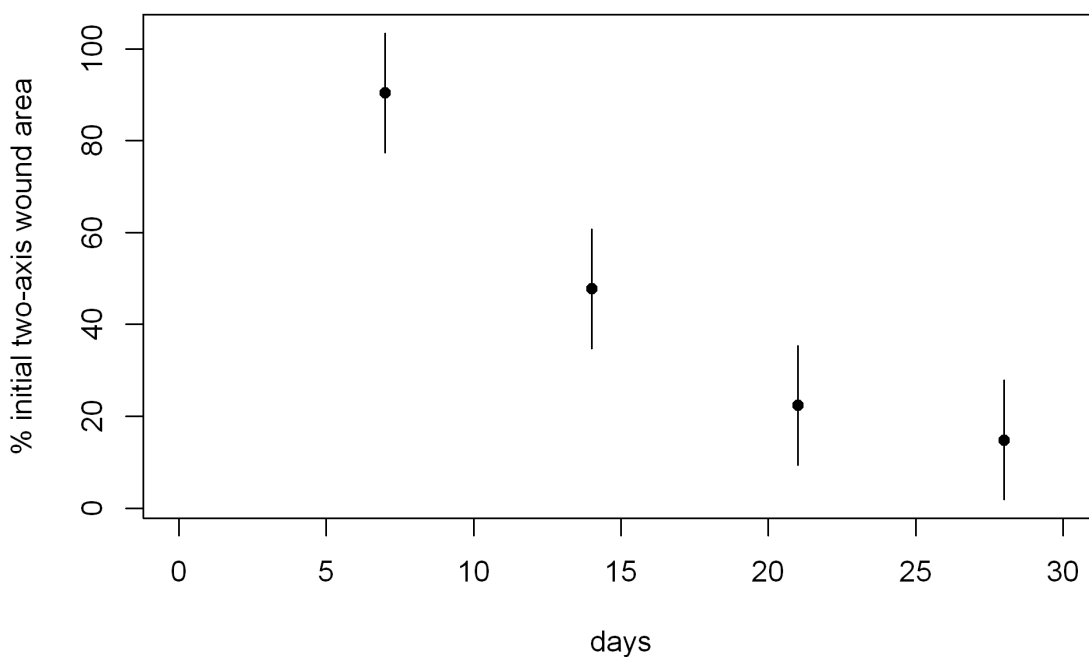


Figure 4. Mean percentage of “two-axis” wound area respect to T0 during the first month of treatment. The mean percentage of wound area at day 7 was 90.41% (95% CI: 77.38 - 103.4), at day 14 was 47.74% (95% CI: 34.72-60.8), at day 21 was 22.38% (95% CI: 9.36 - 35.4) and at day 28 was 14.81% (95% CI: 1.78-27.8) of the original size.

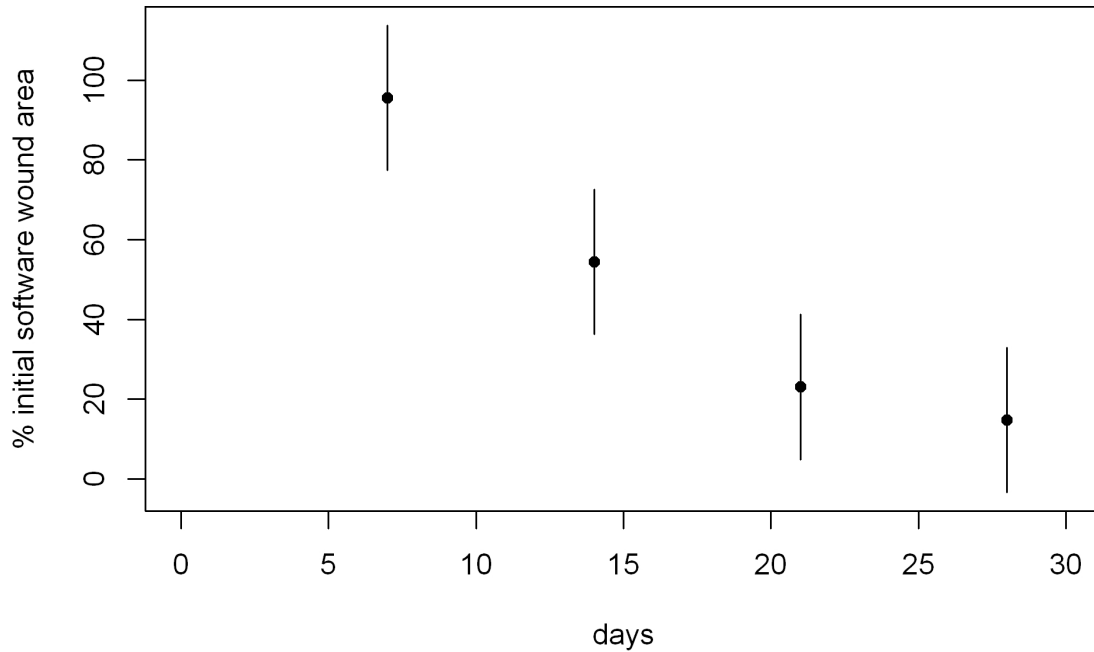


Figure 5. Mean percentage of software wound area respect to T0 during the first month of treatment. The mean percentage of wound area at day 7 was 95.5% (95% CI: 77.33 – 113.8), at day 14 was 54.4% (95% CI: 36.22-72.6), at day 21 was 23.10% (95% CI: 4.89 – 41.3) and at day 28 was 14.79% (95% CI: -3.42-33) of the original size.

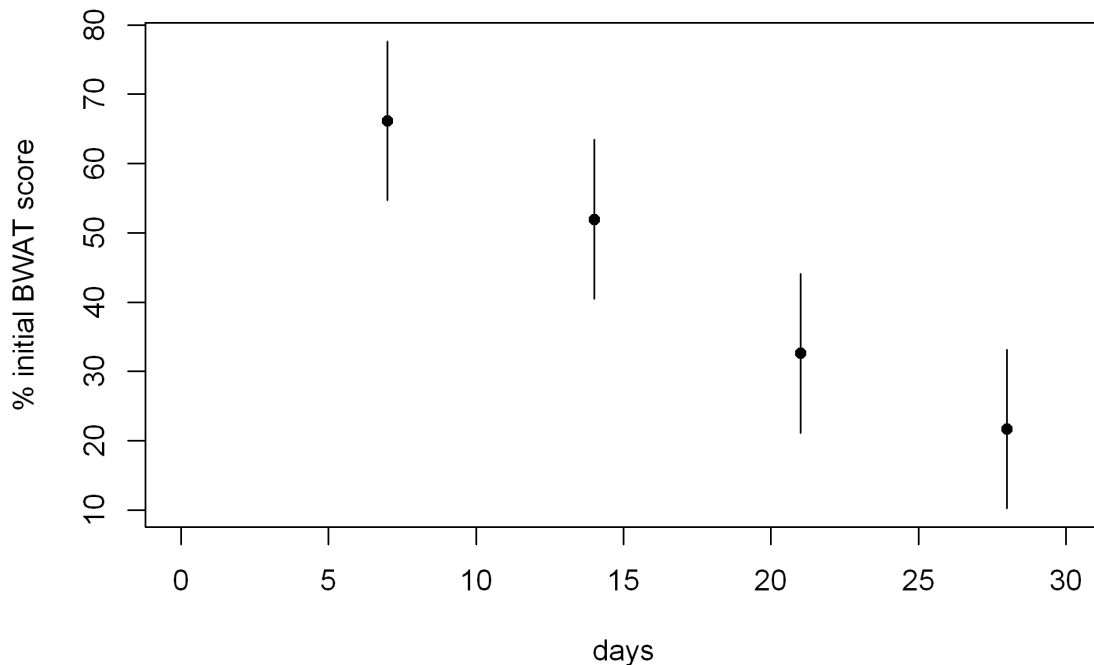


Figure 6. Mean percentage of initial BWAT score during the first month of treatment. The mean percentage of BWAT score at day 7 was 66.15% (95% CI: 54.7-77.6), at day 14 was 51.96% (95% CI: 40.5-63.4), at day 21 was 32.63% (95% CI: 21.1-44.1) and at day 28 was 21.70% (95% CI: 10.2-33.2) of the admission score.

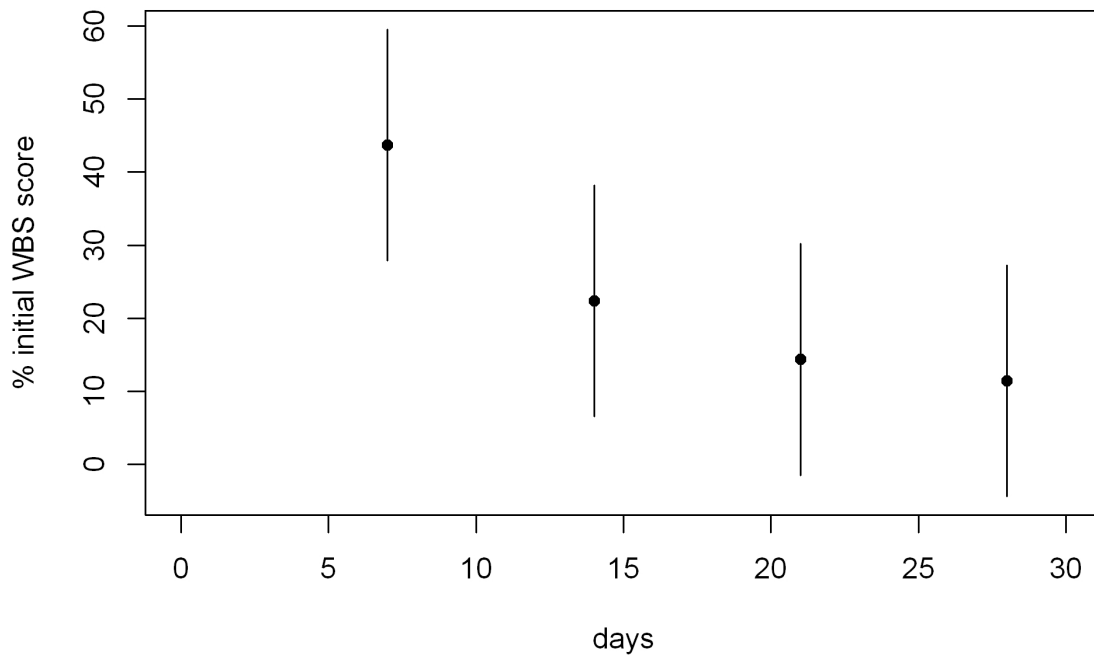


Figure 7. Mean percentage of initial WBS score during the first month of treatment. The mean percentage of WBS score at day 7 was 47.71% (95% CI: 27.86-59.6), at day 14 was 22.38% (95% CI: 6.52-38.2), at day 21 was 14.38% (95% CI: 0-30.2) and at day 28 was 11.43% (95% CI: 0-27.3) of the admission score.

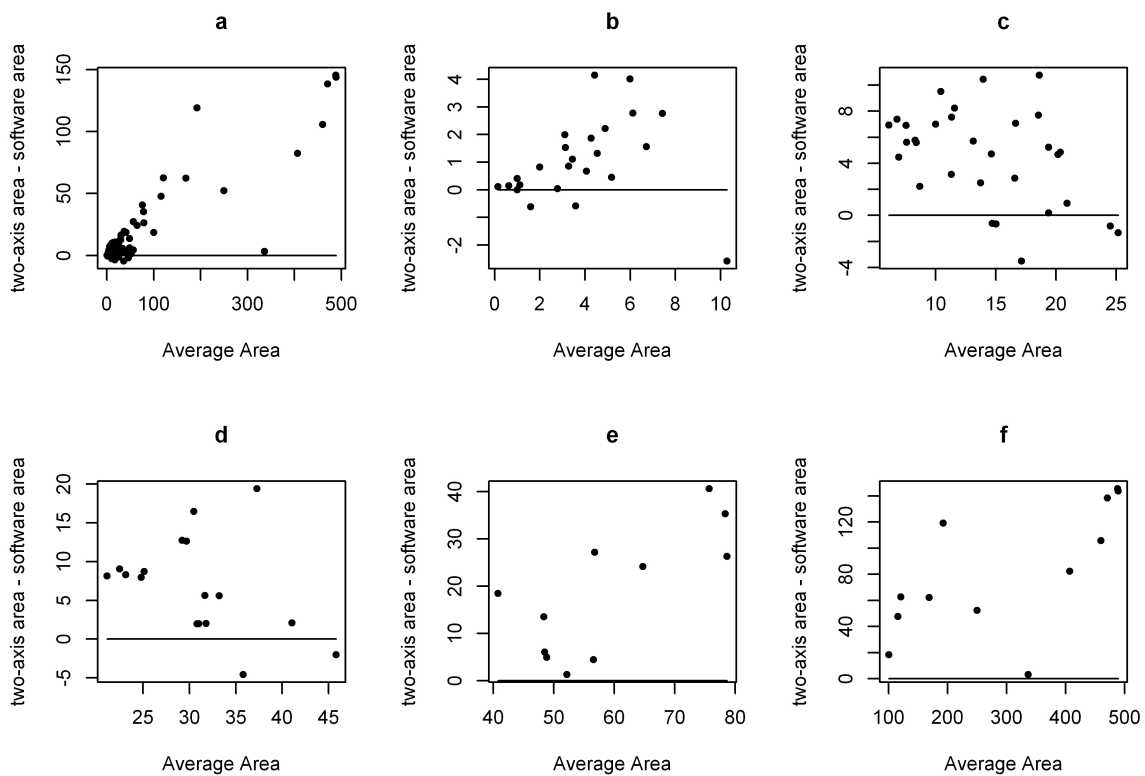


Figure 8. Comparison of area measurement between two-axis and software methods. Panel a: overall wound area measures; panel b: two-axis wound area measures $< 10 \text{ cm}^2$; panel c: two-axis wound area measures $\geq 10 \text{ cm}^2$ and $< 25 \text{ cm}^2$; panel d: two-axis wound area measures $\geq 25 \text{ cm}^2$ and $< 50 \text{ cm}^2$; panel e: two-axis wound area measures $\geq 50 \text{ cm}^2$ and $< 100 \text{ cm}^2$; panel f: two-axis wound area measures $\geq 100 \text{ cm}^2$. An increasing trend for the discrepancy between the two methods with the increasing of the wound area is shown.

WOUND CASE-LOG

Case 3: Traumatic wound on the distal aspect of the forearm.



Fig. 9: A. Wound at time of presentation (T0) after 10 days from the date of the trauma.
B. The same wound after removing of the suture, trichotomy and irrigation.

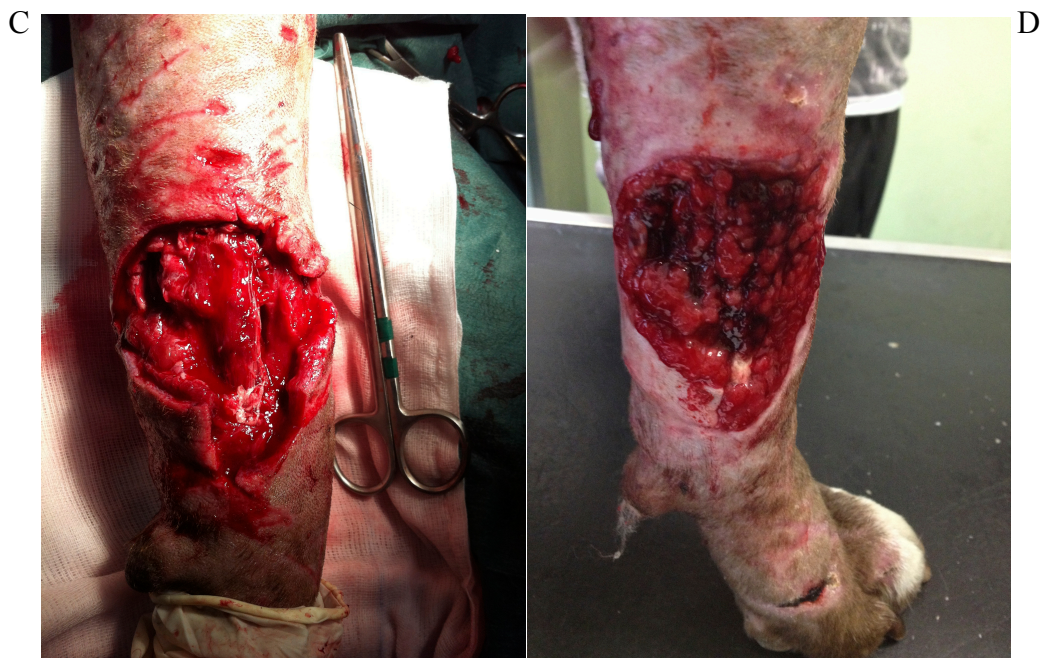


Fig. 9: C. Wound at time of presentation (T0) after surgical sharp debridement.
D. Wound a week later (T8). Granulation tissue began to form. The gauze with partial benzyl ester derivative of hyaluronan were applied.



Fig.9: E. Wound at 23 days after surgical sharp debridement. The wound bed was entirely filled by red granulation tissue. The owner was instructed to daily apply the topical medication.
 F. Wound completely healed (T62).

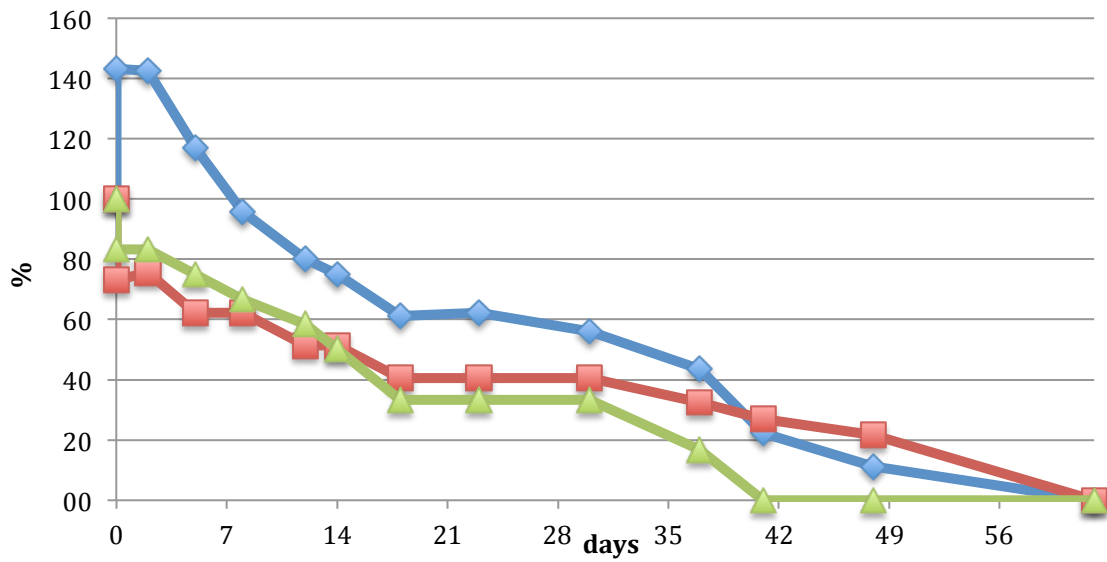


Fig. 9: G. Wound healing assessment for case 3. Wound two-axis area: blue line; BWAT assessment: red line; WBS assessment: green line.

Case 4: Traumatic wound. Ten days before the dog was run down a truck.



Fig. 10: A. Wound at time of admission (T0). The wound was previously treated for ten days with naftalina granules and saline solution irrigation. The dog was hypoalbuminemic at admission. After stabilization of the patient, a surgical sharp debridement was performed.

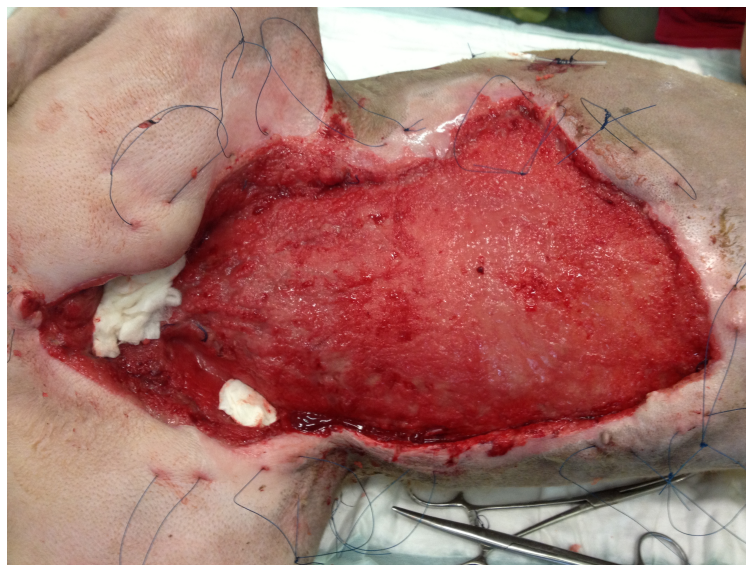


Fig.10 : B. Wound a week after admission (T7). A tie-over bandage was applied to fix the medication in contact with the wound bed. A granulation tissue began to form and the wound edges created by the surgeon during the surgical sharp debridement began to adhere to the underlying tissue. The gauze with partial benzyl ester derivative of hyaluronan were used to fill pocket in the inguinal area.



Fig.10: C. Wound at days 40 (T40). The wound area was decreased. The owners were instructed to daily apply the topical medication. The wound presented a stellate appearance created by the contraction. The wound was considered completely healed 75 days after admission.

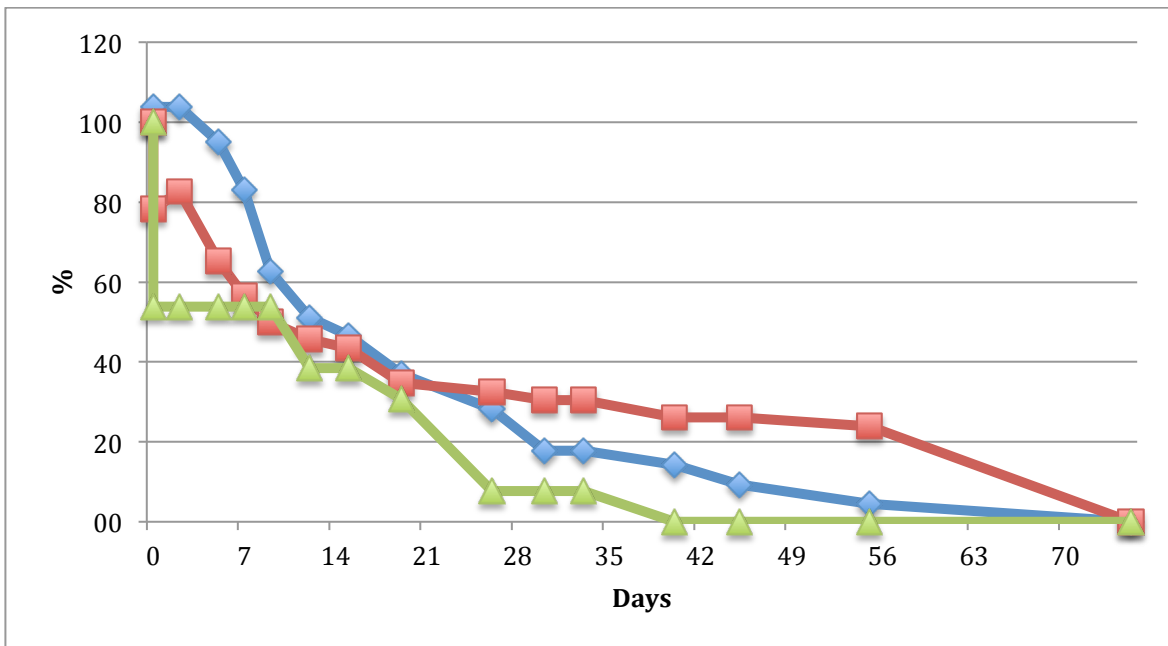


Fig.10: D. Wound healing assessment for case 4. Wound two-axis area: blue line; BWAT assessment: red line; WBS assessment: green line

Case 6 and 7: Traumatic burn wounds generated by electric pad during anesthesia for malignant tumors excision.



Fig. 11: A. Wounds at time of presentation (T0) 14 days after surgical procedure. The hair on the right part of the body of the dog was clipped. A topical daily application of hyaluronic acid plus collagenase-based medication was started to obtain a medical enzymatic debridement.



Fig. 11 B. Wounds after 5 days from the admission. A maturation of the necrotic tissue from a black, dry, adherent eschar to a yellow, non-adherent viable tissue was achieved. A tie-over bandage was used to better fix the medication for the wound of the flank. At that time wounds underwent a surgical sharp debridement to remove all the non-viable tissue.



Fig.11: C. Wounds at days 17. A healthy granulation tissue filled all the wound bed of the flank wound.
 D. The wound on the shoulder was considered healed.



Fig. 11: E. Wounds at days 54. Even the wound on the flank was considered healed.
 Fig. 11: F. Four months after complete healing. An alopecic area with hyperpigmentation of the epidermal tissue was present.

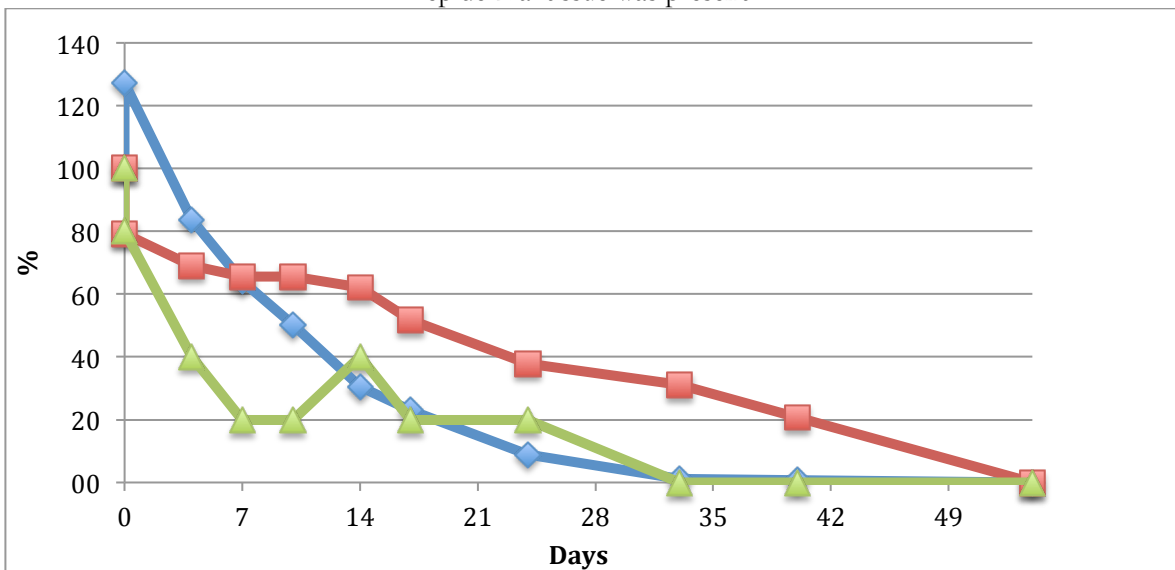


Fig. 11: G. Wound healing assessment for case 6. Wound two-axis area: blue line; BWAT assessment: red line; WBS assessment: green line

Case 8: Excisional wound after failure of a free skin graft. The dog was undergoing chemotherapeutic protocol with vinblastine and prednisone started two weeks before the surgery and continued during wound healing period.



Fig.12 : A. Wounds at admission. A blood clot separated the free skin graft from the underlying tissue.
B. The wound was than healed by second intention.



Fig. 12: C. Wounds a week after admission. Granulation tissue began to form.
Fig.12: D. Granulation tissue completely filled the wound bed.



Fig.12: E. Wounds completely healed at 49 days after admission

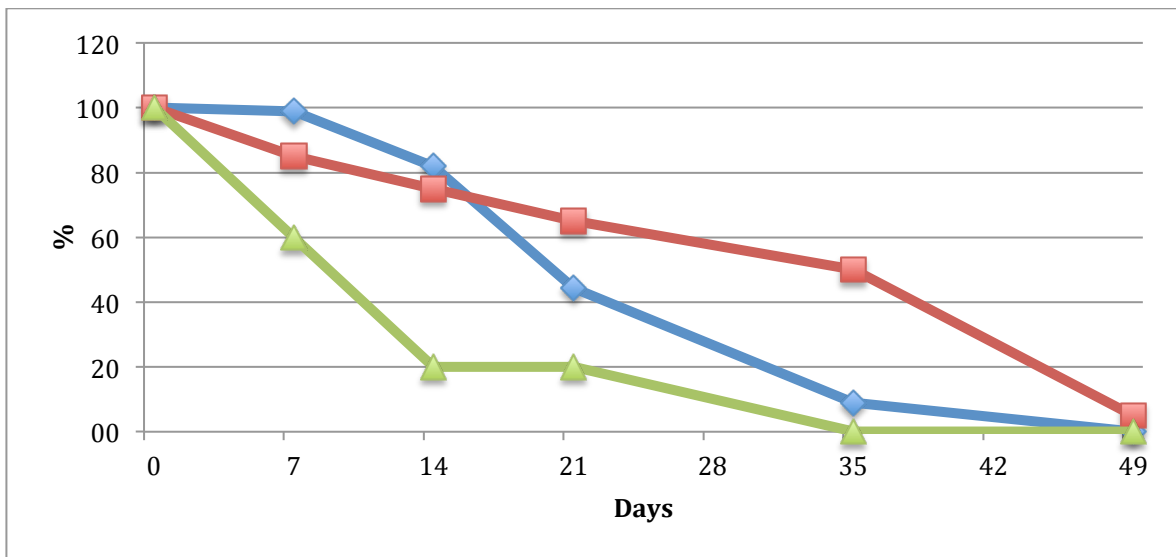


Fig. 12: F. Wound healing assessment for case 8. Wound two-axis area: blue line; BWAT assessment: red line; WBS assessment: green line

CHAPTER 5

Discussion

5. Discussion

The roles of HA in each phase of wound healing have been investigated intensively in human medicine, and HA's properties have recently been successfully used in a number of wound dressing.^{54,61-65,91-93} In veterinary medicine the potential for using HA-based medication on open skin wound in dogs was studied only by two case-control papers in which a cross-linked HA was applied on small (2 cm × 2 cm) experimental open wounds. In those studies controversial results were obtained and also taking into account the positive experience of human medicine, the additional investigation should be reached to increase the knowledge of HA properties also for companion animals.^{6,7} In addition, medical experimental research does not always replicate clinical conditions such as the presence of comorbidities or peripheral tissue damage that impair the healing process as happen instead in all non-experimental wounds making those results' studies potentially no reproducible in the clinical setting.⁹¹ Regarding these issues, the use of HA-containing dressings was evaluated for the first time on a small cohort of non-experimental cutaneous open wounds in client-owned dogs.

The commercial available HA formulations tested in this study were found easy to apply, without the need of intensive staff training and so could be used not only in a veterinary second opinion hospital but even in an ambulatory setting. The formulations in gauze, cream, granules and spray allowed to apply the medication also in the presence of very irregular shape and inside the undermined pocket. The bandages were applied as routine so dogs were able to stay at home and to live a regular life with the exception of takes care of bandages (e.g. T-shirt covering the bandage, Elizabethan collar). Owners were instructed to re-build the bandages only when the wounds were in the late stage

of progression, and no problems were recorded at the weekly examination using this strategy. In fact, the formulations in cream and spray were also described for auto-medication for human beings.

No side effects on wounds and periwounds tissue were recorded in the present study. This finding is in accordance with the past literature in which no side effect and no allergic response were reported for the topical application of HA in wounds, both for dogs and human.^{6,7,61,92,93} Hadley and colleagues histologically proved the absence of inflammatory reaction around the HA-based gel applied on their experimental wounds in dogs.⁷ The unpleasant odor reported by some owners associated with the HA products used in the present study was otherwise tolerated and has been related to the degradation of HA associated to the presence of wound fluid. To bypass the uncomfortable sensation linked to the use of spray, owners were instructed to not use it directly on the wound but to drench sterile gauze and to apply it on the wound.

The frequency between each dressing change varied widely and was mostly influenced by type of dressing and characteristic of the wound at assessment. The adsorbent medication made of partial benzyl ester derivative of hyaluronan was re-applied every about 4 days even if for human medicine this product could be left until for a week. This medication breaks down in contact with wound exudate forming a gel rich of HA. The degradation timing depends on exudate levels and when at the assessment wound achieved a high exudate score the dressing change was scheduled more frequent than weekly. At the same time clinician had to consider this degradation and gel forming when evaluated the type and amount of exudate, especially when the bandage was early changed and the product was not totally adsorbed. It may be possible that this consideration has led to a more frequent dressing change in the first wounds treated respect to the last when a good mastery of the product was achieved.

One factor that should be taken into account in the choice of dressing is the cost-benefits ration and even, for veterinary practitioners, the easy availability also for not hospital facility. The HA-containing dressings used in this study are available in any drug store of the country and have a cost range from of about 30 (gauze, 10 cm × 10 cm) to 10 euro (15 g cream tube) at which should be added the regular cost for bandage materials and clinical control. Obviously, the cost increases with increasing size of treated area and with the need of more than one product. On the contrary, the increased cost of an advance medical dressing has to be set against a shorter period of treatment and also to the lack of need of hospitalization for the type of medications used in this study.

In front of these good results is mandatory to achieve also a good wound outcome. To test the outcome of a wound medication a control group is always necessary. The explorative intent of this study, the small number of the sample and the difficult to build a control group that overlaps the characteristic of non-experimental wounds and patients allow to extrapolate only observational data. Authors considered the TWH (of about 1 month) achieved with HA-based dressing in canine non-experimental open wound a promising result. In particular, the wound area reduction was higher between 7 and 14 days with a mean wound area at 2 weeks halved than admission (Figures-4 and -5). Recently, the use of negative pressure in spontaneous wounds healed only by the second intention achieved a mean healing time of 26 days while excisional wound without advance dressing achieved a median healing time of 53 days.^{5,94} Nevertheless due to the specific nature and features of spontaneous injuries any comparison with other studies that used different dressing or therapy involves possible high-risk bias and confirmation in further studies of the impact of the HA-medications on wound outcome is necessary.

Reading the literature on HA and wound healing seemed that HA plays a role in each phases of the healing process. Many studies found that HA not only

enhance the fibroblast and vascular deposition in the granulation tissue, but also the keratinocyte proliferation during epithelialization.⁵⁶⁻⁶⁰ Due to ethical implication and the clinical nature of this study no incisional biopsy of the healing tissues was sample during the present study, so was impossible to better elucidate the structure of the newborn tissues, and in addition in absence of a control group to have a comparison with no treated wounds. Otherwise, to authors' experience and observation the granulation tissue filled the wound gap in a very quick period and generally grossly appeared of a very good and healthy quality making supposed that the HA have principally promoted the deposition of fibroblast and neo-angiogenesis. This is also reported by another experimental study on HA in dogs, in which authors described a better gross appearance of the granulation tissue for the treated wounds.⁶

Periodic wound assessment to document healing progression and effectiveness of treatment is one of the most important parts of wound management. Wounds were reassessed at every bandage change. In human medicine, the frequency of assessment is often related to specific patient and wound, with a higher frequency for acute than chronic wound.⁸² In veterinary experimental research with HA the wounds were re-assessed every 3 days or weekly but no information on clinical setting is available.^{6,7} In author's experience with these HA-based products the assessment may be generally repeated after a period from 4 to 7 days to detected noticeable alteration that justify to declare an improvement or worsening of the wound even if the clinician as to change early the dressing as happen in case of dogs prone to remove it.

Wound dimensions are the most frequent used parameters for healing evaluation in clinical and research settings.^{7-9,11,80}[8-10,13,27]. Many are the possible measures to evaluate a dimensional assessment.⁸¹ In veterinary medicine, the majority of the studies referred to area for measuring the wound extension.^{7-9,11} In experimental setting often digital imaging and software application have been

used while linear metric dimension and mathematical formula have been used in clinical research.^{7-9,94} Obviously, the technique to calculate the wound area as a rectangular only approximates the real wound area that could be of irregular shape, but it was made because was the simplest, least expensive, reliable and fast method in a clinical setting.⁸¹ On the other hand, this could have overestimated the real size of about 25% in case of circular wound area.⁸¹ In fact, the two-axis methods generally represent a bigger wound area respect to software assessment also in this present study. However, this method was always applied, and the possible error was repeated during the assessments making probably correct the comparison of two evaluations in the same wound. To be more precise even the software was applied. This software was free, easy to use and the only necessary equipment is a digital camera and a personal computer. However, the authors find some possible bias use it when the extension of the wound followed the natural curves of the body (e.g. wound extended for more than 180° around extremity) and the bi-dimensional picture achieved was not able to represent the real extension. In these cases, the technique reported by Tambella *et al.* where the perimeter of the open wound area was captured by hand with a fine tip permanent marking pen on a transparent sheet placed directly on the wound bed and then photographed and measured with the software should be use.¹² Taking in consideration the difference achieved with this two methods (Table-3) and the absence of a gold standard, the method chooses at the first assessment should be repeated during the healing process, at least until the wound became small (<9 cm²) and the difference could be more probably <1 cm².

The use of wound area alone in the assessment of wound healing progression has the disadvantage to do not take into account the change in the quality of the wound bed that not always reflect a change or a reduction in wound dimensions.⁸⁰ In veterinary medicine no data are available concerning this kind

of assessment even if some authors discussed about the addition in wound judgment of other parameters, such as fluid characteristics, granulation tissue, periwound, status and hidratation to subjectively evaluated experimental wound in dogs.^{7,11,12} Considering this, two visual wound scales previously used in human medicine were parallel applied at each assessment. In human medicine this kind of evaluations were very diffuse and even if a real gold standard method is not approved, these tools represent an active part of wound management.^{80,77,95} These scales are generally used and drawn for the evaluation of the most frequent human chronic wounds and their application on an acute open wound in dogs remained an explorative attempt.

In addition, the weight of the different tool characterizing the wound at the assessment reflect the mutability of the healing process and is unequivocally linked to the selection of appropriate dressing that should be based on the type of wound, healing status, bio-burden present and amount of exudate.^{77,96} In this scenario, wound quality assessment is also performed to determine if wound is changing, not only because it actually may be healing, but also because changes provide useful tool to improve the plan of care and direct clinical decision-making.⁸⁷ In this case series the scores, in association with manufacture's instruction linked to 'TIME approach, were used to direct decision about which HA-containing dressing should be apply. The 'TIME approach is based on the goal to remove local barriers to healing and optimizes the tissue environment to achieve wound healing. This concept of wound bed preparation in human medicine initially arose out of a need to address chronic wound but many wounds that fall into the traditional classification of "acute" could be amenable to management with the wound bed preparation concept.⁷⁷ The 'TIME concept of human medicine, although not specifically studied in the veterinary field, partially overlaps wound care guidelines reported for animal and are also reported in animal wound care book.^{1,97}

The best BWAT score for all wounds was obtained the same date of the total epithelialization while the best WBS score was achieved before. This result is justified by the fact that WBS score was delineated in human chronic wound to predict ultimate wound closure and not to assess the completion of the process as BWAT does, instead.⁹⁰ After the obtainment of the best WBS score, all the 12 wounds progressively healed until complete epithelialization. In addition using both scales, the greatest reduction of the score was obtained in the 1st week with a mean decrease of 66.3% for WBS and 33.8% for BWAT respect to the admission scores. These results confirmed that qualitative assessment was especially useful in the early aspects of the healing of the acute wound in dogs when often the size of the wound do not change.⁸⁰ Furthermore, the wound that received debridement, even medical or surgical, resulted in a larger size that taking into account only this data could not revealed an improvement of the quality of the wound as instead confirmed by both scoring systems (Table-2).

In both scales many are the parameters considered to elaborate the final score. In human seems that exudate control, minimal eschar and decrease size have a higher prognostic impact on wound healing of chronic wound respect to other such as quality of granulation tissue.^{87,98} The low number of cases in this study did not permit to analyze which parameters including in the scales could have the higher impact on second intention wound healing in acute wounds in dogs. The BWAT scale takes into account more parameters than the WBS and nowadays this could allow considering it more complete even taking into account the higher agreement achieved by two different operators. However, further studies are needed to delineate which parameters are more useful to describe the healing process in dogs and which are secondary or even unnecessary.

CHAPTER 6

Conclusion

6. Conclusion

The Hyaluronic Acid-containing dressings used in this study are easy to apply, well tolerated and carry promising results in improving healing of acute open wounds in dogs. In this explorative study the investigation of prognostic significances of site and size of the wound on healing time was not possible and further studies are necessary, also taking into account the comparison between different dressing and therapy on healing rate and on cost-benefit ratio. The use of scale scoring systems to assess wound healing gives consistent results complimentary to wound area reduction and the application of this assessment tools should be evaluated in veterinary medicine. The therapeutic approach based on quality wound assessment used in this study could be considered a primary pilot systemic decision-making process easy to follow wound treatment routine with HA-based dressing in daily veterinary clinical practice.

CHAPTER 7

References

7. References

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