SOFIA CICOLO DA SILVA

Clinical and histopathological evaluation of tilapia skin (*Oreochromis niloticus*) as an occlusive biological curative in equine wounds

São Paulo
2022

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Dissertation submitted to the Postgraduate Program in Veterinary Surgical Clinic of the School of Veterinary Medicine and Animal Science of the University of São Paulo to obtain the Master's degree in Sciences.

Department:

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Prof. Dr. André Luis do Valle De Zoppa

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Comissão de Ética no Uso de Animais

CERTIFICADO

Certificamos que a proposta intitulada "AVALIAÇÃO CLÍNICA E HISTOPATOLÓGICA DO USO DA PELE DE TILÁPIA (OREOCHROMIS NILOTICUS), COMO CURATIVO BIOLÓGICO OCLUSIVO EM FERIDAS EM EQUINOS NA ROTINA CLÍNICA DO HOVET USP ", protocolada sob o CEUA nº 3731111218 [illegible], sob a responsabilidade de **André Luís do Valle de Zoppa** *e equipe; Sofia Cicolo da Silva* - que envolve a produção, manutenção e/ou utilização de animais pertencentes ao filo Chordata, subfilo Vertebrata (exceto o homem), para fins de pesquisa científica ou ensino - está de acordo com os preceitos da Lei 11.794 de 8 de outubro de 2008, com o Decreto 6.899 de 15 de julho de 2009, bem como com as normas editadas pelo Conselho Nacional de Controle da Experimentação Animal (CONCEA), e foi **aprovada** pela Comissão de Ética no Uso de Animais da Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo (CEUA/FMVZ) na reunião de 14/12/2018.

We certify that the proposal "EQUINOS NA ROTINA CLÍNICA DO HOVET USP CLINICAL AND HISTOPATHOLOGICAL EVALUATION OF USING TILAPIA SKIN (OREOCHROMIS NILOTICUS) AS AN OCCLUSIVE BIOLOGICAL CURATIVE IN EQUINE WOUNDS IN THE CLINICAL ROUTINE OF HOVET USP", utilizing 20 Equines (males and females), protocol number CEUA 3731111218 [illegible], under the responsibility of **André Luís do Valle de Zoppa** *and team; Sofia Cicolo da Silva* - which involves the production, maintenance and/or use of animals belonging to the phylum Chordata, subphylum Vertebrata (except human beings), for scientific research purposes or teaching - is in accordance with Law 11.794 of October 8, 2008, Decree 6899 of july 15, 2009, as well as with the rules issued by the National Council for Control of Animal Experimentation (CONCEA), and was **approved** by the Ethic Committee on Animal Use of the School of Veterinary Medicine and Animal Science (University of São Paulo) (CEUA/FMVZ) in the meeting of 12/14/2018.

Finalidade da Proposta: Pesquisa

Vigência da Proposta: de 12/2018 a 12/2021 Área: Clínica Cirúrgica Veterinária

Origem:	Animais de proprietários						
Espécie:	Equídeos	sexo:	Machos e Fêmeas	idade:	0 a 50 anos	N:	20
Linhagem:	qualquer uma			Peso:	0 a 1000 kg		

Local do experimento: HOVET USP

São Paulo, 19 de fevereiro de 2019

Profa. Dra. Anneliese de Souza Traldi
Presidente da Comissão de Ética no Uso de Animais
Faculdade de Medicina Veterinária e Zootecnia da Universidade de São Paulo

Roseli da Costa Gomes
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EVALUATION FORM

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Date: _____/_____/_____

Committee Members

Prof. Dr.____

Institution:_____________________________Decision:_________________________

Prof. Dr.____

Institution:_____________________________Decision:_________________________

Prof. Dr. ____

Institution:_____________________________Decision:_________________________

DEDICATION

(To my family, friends, professors and pets that helped me during my path).

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“Happiness can be found in even the darkest of times, if one only remembers to turn on the light.”

J.K.Rowling

RESUMO

CICOLO, S.S. **Avaliação clínica e histopatológica do uso da pele de tilápia (*Oreochromis niloticus*) como curativo biológico oclusivo em feridas em equinos:** 2022. 70. p. Dissertação (Mestrado em Ciências) - Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2022.

A pele de tilápia do Nilo tem sido usada em feridas provocadas por queimaduras em humanos e animais selvagens, apresentando ótimos resultados na cicatrização e também na redução da dor dos pacientes. Além do mais, a pele de tilápias é de baixo custo aquisitivo por ser considerada subproduto. O objetivo deste estudo foi avaliar a pele de tilápia do Nilo como curativo oclusivo em equinos, as consequências do seu uso no processo de cicatrização, de forma clínica e histopatológica. Foram utilizados sete equinos, com onze feridas que não puderam ser fechadas por primeira intenção, a maioria delas crônicas. Foram incluídos neste estudo equinos, dos dois gêneros e de todas as raças. As feridas foram mensuradas, fotografadas, biopsiadas, limpas e, por fim, foi realizada a troca do curativo de pele de tilápias semanalmente durante 28 dias. Amostras de sangue dos pacientes foram coletadas em tubos com EDTA para realização do hemograma, leucograma e fibrinogênio. O cálculo da área das feridas foi estabelecido com o programa Image J para verificar a porcentagem de contração das feridas. Observou-se que na segunda semana de tratamento há grande presença de secreção densa e amarelada, compatível com o intenso infiltrado neutrofílico no mesmo período. As feridas crônicas apresentaram processo secretivo, tornando-se feridas ativas, alterando os mecanismos de formação do tecido de granulação exuberante para a formação de um tecido cicatricial efetivo. Este curativo permitiu a diminuição do número de trocas para uma vez por semana, resultando em diminuição do estresse e da dor dos animais, e também do custo, devido ao menor número de bandagens utilizadas. Conclui-se que a pele de tilápia do Nilo, utilizada como curativo oclusivo, durante os 28 dias, melhora significativamente o processo de cicatrização com redução importante na área da ferida, acelera a cicatrização e induz resposta inflamatória aguda nas lesões dos equinos.

Palavras-chave: Cavalos. Enxerto. Regeneração cutânea. Pele de peixe.

ABSTRACT

CICOLO, S.S. **Clinical and histopathological evaluation of tilapia skin (*Oreochromis niloticus*) as an occlusive biological curative in equine wounds**. 2022. 70 p. Dissertação (Mestrado em Ciências) – Faculdade de Medicina Veterinária e Zootecnia, Universidade de São Paulo, São Paulo, 2022.

Nile tilapia fish skin (NTFS) had been used in humans and wild animals to heal wounds and showed great results on healing process, and also on patient pain control. Also NTFS is easy to access due to it being an industrial waste. The objective of the present study was to evaluate NTFS used as an occlusive curative, the consequences on wound healing process clinical and histopathologically. Seven equines, presenting 11 wounds that couldn't be healed through a first intention, were used in this study. Most wounds were chronic. Equines of both gender and all breeds were included in this study. The wounds were measured weekly, photographed using a centimeter scale, biopsied for histopathological analysis, cleaned and tilapia curative was changed for 28 days. EDTA tube blood sample was collected for red blood cell, leukocytes and fibrinogen evaluation. Image J software was used to measure wound area and calculate wound contraction percentage. During the second week of treatment an intense yellow secretion has been seen, compatible with intense neutrophilic infiltrate observed on histopathological at the same period. Chronic wounds start the secretion process, changing healing pathways from exuberant granulation tissue (EGT) formation to a repair process and effective cicatrization tissue formation. This curative allowed reduced changes from every two days to once a week. This implies decreasing animal stress, pain and treatment cost, as a smaller amount of bandages is necessary. NTFS used as an occlusive curative significantly improves healing process during 28 days treatment, presenting significant reduction on wound area, speeding up healing process and induces acute inflammatory response (AIR) in equine wounds.

Keywords: Horse. Graft. Skin Regeneration. Fish skin.

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ABBREVIATION LIST

RHL	Right Hind limb
LHL	Left Hind Limb
LFL	Left Forward Limb
RFL	Right Forward Limb
EGT	Exuberant Granulation Tissue
ROS	Reactive Oxygen Species
ATB	Antibiotics
NTFS	Nile Tilapia Fish Skin
FDA	US Food and Drug Administration
AIR	Acute Inflammatory Response
CM^2	Square centimetre
CM	Centimetre

SUMMARY

1 TILAPIA SKIN (OREOCHROMIS NILOTICUS) AS AN OCCLUSIVE BIOLOGICAL CURATIVE IN EQUINE WOUNDS: SHORT COMMUNICATION [1]

1.1 ABSTRACT/RESUMO

Tilapia skin is currently used in humans and wild animals that present burning wounds, demonstrating a great result. The objective of this study is to evaluate if tilapia skin used as an occlusive curative improves equine wound healing in two present with chronic wound. Both animals are males, adults, both of breed Mangalarga Marchador South America. Every seven days we measured, photographed, biopsied for histopathological analysis, cleaned and changed the tilapia curative. Image J software was used to measure wound area. Tilapia skin as an occlusive biological factor seemed to improve healing process, wounds present an area reduction and clinical improvement during 35 days treatment, even though it is still waiting for complete wound healing. In equine tilapia skin curative seemed to speed up the healing process and allowed reduced curative change from every two days to once a week. This implies decreasing animal stress, less pain and treatment cost reduction since we used fewer bandages. Beside that, tilapia skin is seen as industrial waste. Furthermore, it avoids using antibiotics, which reduces environmental pollution and there´s no antibiotic resistance issues.

Keywords: Horse. Graft. Skin Regeneration. Fish skin

Resumo

A pele de tilápia está sendo utilizada em humanos e animais silvestres com feridas por queimadura demonstrando um excelente resultado. O objetivo do estudo é avaliar se a pele de tilápia utilizada como curativo oclusivo melhora o processo de cicatrização em dois equinos machos adultos da raça Mangalarga Marchador, os quais apresentam feridas crônicas. A cada 7 dias as feridas eram medidas, fotografadas, biopsiadas para a análise histopatológica, limpas e o curativo de pele

[1] Silva SC, Ríspoli VFP, Graner C, Sá LRM, Belli CB, Zoppa ALV. Using tilapia skin (Oreochromis niloticus) as an occlusive biological curative in equine wounds. Short communication. Braz J Vet Res Anim Sci. 2019;56(4):e154079. https://doi.org/10.11606/issn.1678-4456.bjvras.2019.154079

de tilápia trocado. O programa Image J foi utilizado para calcular a área da ferida. A pele de tilápia utilizada como curativo oclusivo parece ter um efeito positivo na cicatrização das feridas, a área diminuiu e o aspecto clínico melhorou nos 35 dias avaliados, no entanto é necessário esperar pela cicatrização completa das feridas. Em equinos, a utilização de curativo de pele de tilápia parece diminuir o tempo de cicatrização e permite a redução do número de trocas de curativos de a cada dois dias para uma vez por semana. Isso implica em menos estresse e dor para o animal devido à menor manipulação e menor custo de tratamento, pois há menor quantidade de material de curativo sendo utilizado. Além disso, permite evitar a utilização de antibióticos, o que diminui o impacto ambiental e não gera resistência.Palavras-chave: Cavalos. Enxerto. Regeneração cutânea. Pele de peixe.

1.1.2 SHORT COMMUNICATION

Horses frequently suffer traumatic wounds. They can become chronic wounds, presenting delay and complication to heal especially in distal extremities. A considerable number of animals have their athletic career jeopardized because of persisting lameness, swollen limbs, exuberant granulation tissue and extensive scars. Although primary or delayed closure is preferred as treatment, it’s not always possible, in cases of unmanageable contamination, excessive tissue loss or severe compromise of the tissue, wound healing by second intention is often the only option (Caron, 1999; Hendrickson & Virgin, 2005; Wilmink & Van Weeren, 2005).

The local environment exerts an important impact on wound repair. Exuberant granulation tissue (EGT) appears to be influenced by inflammatory mediators. In equine dermal healing, a weak persistent inflammatory response occurs, which is more pronounced in the extremities than in the trunk. EGT develops more in limb wounds and spare body wounds. Occlusion of the microvasculature suggests that the presence of relative tissue hypoxia is detrimental to the inflammatory response and encourages excessive angiogenesis and fibroproliferation via cytokines and other mediators (Theoret et al., 2013).

Neutrophils and macrophages infiltrate the wound and are thought to help clear the wound and produce cytokines (Mirza et al., 2009). Leukocytes in ponies produce more reactive oxygen species (ROS) than horses, which is necessary for bacterial killing. The low initial production of TNFα, IL-1, chemoattractants, TGF-ß, and ROS in horses can explain the weak onset of the inflammatory response and the ensuing persistence of inflammation (Wilmink & Van Weeren, 2005).

Recently, fish collagen has gradually attracted attention because of its abundance and low price. Tilapia skin collagen sponge and electrospun nanofibers were developed for wound dressing. It was composed of at least two α-peptides. They had good thermal stability and swelling properties and could significantly promote the proliferation of human keratinocytes and stimulate epidermal differentiation through the up-regulated gene expression of involucrin, filaggrin, and type I transglutaminase in human keratinocytes. The collagen nanofibers could also facilitate rat skin regeneration (Zhou et al., 2016). It also showed a good result in burn wounds in humans (Lima-Júnior, 2017).

Amino Acid Composition (MCPs) from fish differs greatly from those from terrestrial livestock in physicochemical properties, they also present unique physiological functions including antibacterial, antioxidant, antihypertensive, neuroprotective and anti-skin-aging activities. MCPs from the skin of tilapia contained seven essential amino acids and ten nonessential amino acids. Collagen hydrolysates usually contain a high concentration of collagen tripeptides (Hu et al., 2017).

Fresh tilapia skin was donated by a fisher farm. The samples were emerged in aqueous chlorhexidine 0.2% for 20 minutes, wrapped in gauze and then frozen. To prepare the tilapia skin for use, the samples were rehydrated using sterile saline solution.

The present study is the first reporting using tilapia skin as a wound dressing, it was applied in two horses that presented chronic wounds. Animals are sedated using xylazine 10% and diagnostic biopsy (6 mm diameter) was collected every 7 days, wounds were measured and area calculated by Image J software (Table 1). Results showed habronemiasis and EGT on the first animal biopsy and EGT and eosinophilic dermatitis on the second patient. Animal 2 presented wounds only in limbs and the second one had wounds on both pelvic limbs and back. EGT on animal 1 was resected with a scalpel blade and oral ivermectin was administered. All wounds were cleaned with sterile saline solution containing gentamicin (1.6 g/L) and gauze. Tilapia skin was applied in both cases covering the whole area of the wound. The following procedure involved the bandage of the wound with orthopedic cotton and vet wrap. Diaper and tape were used on the recovery to keep the moisture of the tilapia skin for the next 7 days.

After 3 days, the bandage from animal 2 left limb was changed, because he had bitten it. After seven days, the wounds from animal number 2 were healthy pink, there was a fibrin layer recovering and EGT had disappeared. There was a considerable reduction in wound size. The same bandage changing protocol proceeded, repeated using saline solution only. Animal 1 did not show the same progress due to the lack of contact between the horse's skin and the tilapia skin created by blood as the result of the EGT resection.

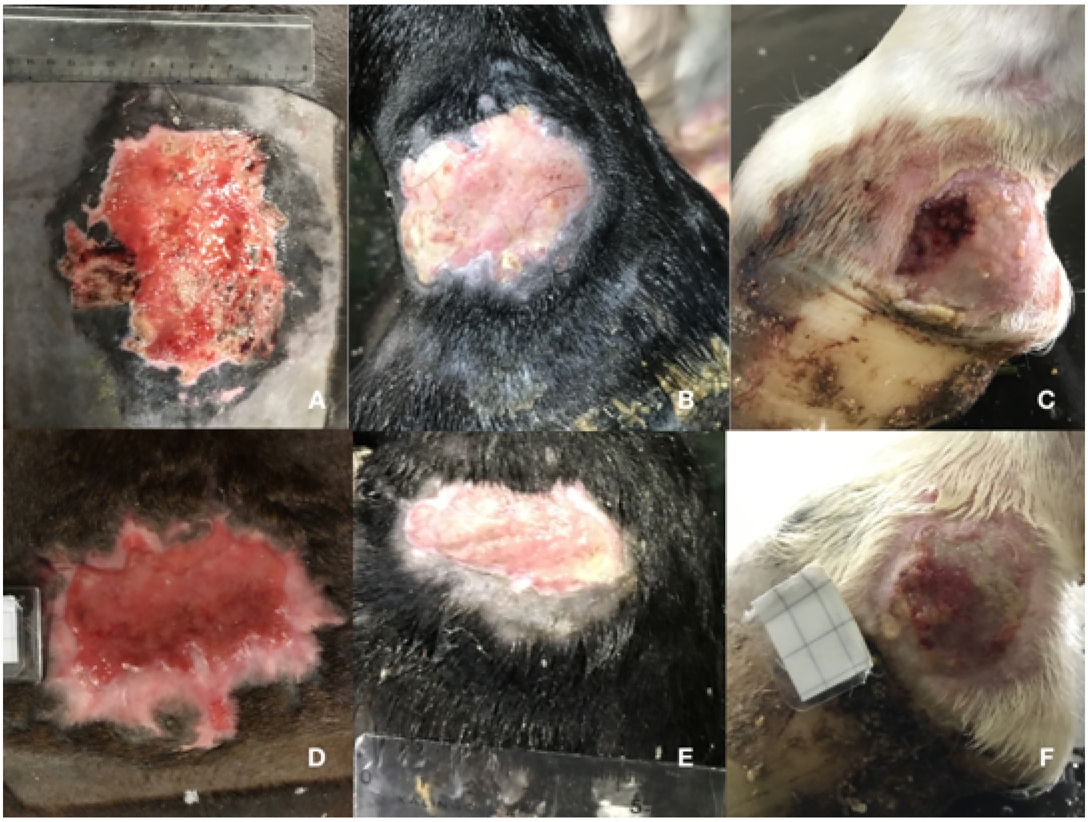
Presence of fur in the healing region near the edges was noticed after 14 days of evolution. The middle of the wound was healthy pink and a great large area of the edges of the wound was epithelialized in both cases. The same bandage changing protocol from day 7 was repeated. A great reduction in wound size, on Table 1, along with surface epithelization was noticed after 28 days of treatment, as shown in Figure 1 to 3.

Table 1 - Area of wound before applying tilapia skin and after 35 days.

	Wound 1 A	Wound 1 B	Wound 2 A	Wound 2 B	Wound 2 C
Area Before(cm)	25	31.8	110.47	6.29	12.164
Area 35 Days (cm)	4	19	53	2.8	10.4
Percentage wound reduction %	84	40.3	52.1	55.5	14.6

(A) Animal 1 front limb wound before applying tilapia skin; (B) Animal 1 front limb wound after 42 days using tilapia skin bandage; (C) Animal 1 pelvic limb wound before applying tilapia skin; (D) Animal 1 pelvic limb wound after 42 days using tilapia skin bandage.

Figure 1 - Animal 1 front limb wound treated with tilapia fish skin



(A) Animal 2 back wound before applying tilapia skin; (B) Animal 2 back wound after 35 days using tilapia skin bandage; (C) Animal 2 right pelvic limb wound before applying tilapia skin; (D) Animal 2 right pelvic limb wound after 35 days using tilapia skin bandage; (E) Animal 2 left pelvic limb wound before applying tilapia skin; (F) Animal 2 left pelvic limb wound after 35 days using tilapia skin bandage.

Figure 2 – Animal 2 back wound treated with tilapia fish skin

Figure 3 - (A) Preparing frozen tilapia skin to apply; (b) tilapia skin applied in animal's 1 back wound

Our findings indicate that tilapia skin seems to improve wound healing in chronic wounds and it decreases EGT formation with no collateral effects, even though a complete histopathological analysis is still needed as well as waiting for complete wound healing. No ATB was administered during treatment. Furthermore, in our routine, wound bandages are usually changed every 2 days, so a lot of material was spared, since we changed it every 7 days.

1.1.3 References

1 Caron JP. Management of superficial wounds. In: Auer JA, Stick JA, editors. Equine surgery. Philadelphia: W. B. Saunders; 1999. p. 129-40. (vol. 1).

2 Hendrickson D, Virgin J. Factors that affect equine wound repair. Vet Clin North Am Equine Pract. 2005;21(1):33-44. http://dx.doi.org/10.1016/j.cveq.2004.11.002. PMid:15691598.

3 Hu Z, Yang P, Zhou C, Li S, Hong P. Marine collagen peptides from the skin of Nile Tilapia (Oreochromis niloticus): characterization and wound healing evaluation. Mar Drugs. 2017;15(4):102. http://dx.doi.org/10.3390/md15040102.PMid:28358307.

4 Lima-Júnior EM. Uso da pele de tilápia (Oreochromis niloticus), como curativo biológico oclusivo, no tratamento de queimaduras. Rev Bras Queimaduras. 2017;16(1):7-10.

5 Mirza R, Dipietro LA, Koh TJ. Selective and specific macrophage ablation is detrimental to wound healing in mice. Am J Pathol. 2009;175(6):2454-62. http://dx.doi.org/10.2353/ajpath.2009.090248. PMid:19850888.

6 Theoret CL, Olutoye OO, Parnell LK, Hicks J. Equine exuberant granulation tissue and human keloids: a comparative histopathologic study. Vet Surg. 2013;42(7):783-9. http://dx.doi.org/10.1111/j.1532-950X.2013.12055.x. PMid:24015864.

7 Wilmink JM, Van Weeren PR. Second-intention repair in the horse and pony and management of exuberant granulation tissue. Vet Clin North Am Equine Pract. 2005;21(1):15-32. http://dx.doi.org/10.1016/j.cveq.2004.11.014. PMid:15691597.

8 Zhou T, Wang N, Xue Y, Ding T, Liu X, Mo X, Sun J. Electrospun tilapia collagen nanofibers accelerating wound healing via inducing keratinocytes proliferation and

differentiation. Colloids Surf B Biointerfaces. 2016;143:415-22. http://dx.doi.org/10.1016/j.colsurfb.2016.03.052. PMid:27037778.

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2 NILE TILAPIA FISH SKIN AS AN OCCLUSIVE BIOLOGICAL BANDAGE IN EQUINE WOUNDS[2]

2.1 Introduction

Equine wounds have always been a challenge for equine veterinarians. Nowadays we understand that immunologic characters of this species contribute to the long and troubled healing process. Chronic wounds may take a long time to heal, and are still an important percentage of the reason why horses are hospitalized. Frequently, surgical procedure is necessary to remove exuberant granulation tissue and long hospitalization. Expenses involved may be an impediment for the owners. Occlusive curatives have been recently described as a good option for equine EGT treatment (1–3).

Nile Tilapia Fish Skin (NTFS) has shown a great result when used as occlusive bandage in burned humans and animals. Furthermore, it's an abundant and cheap material, easily found in Brazil and around the world (4,5).

For human burning wounds, there is a gold standard treatment (sulfadiazine cream treatment) comparable with tilapia skin. In equine chronic wounds, however, there is no gold standard treatment yet (1,6,7).

Exuberant granulation tissue can lead to scar formation, compromising mobility and jeopardizing equines` athletic career. Clinical and histopathological evaluation may help to define how tilapia skin is involved in the healing process, and the aspect of final results in the treatment of equine wounds (1,2,8).

2.2 Literature Review

Horses frequently suffer traumatic wounds and healing is often delayed and complicated when compared to other species. Primary closure is prefered as treatment, but it's not a choice when the wound is contaminated or in chronic wounds (9,10). Unfortunately, in some horses, granulation tissue proliferation continues unabated, leading to exuberant granulation tissue (EGT). EGT

[2] Submitted to Braz J Vet Res Anim Sci.

characteristically is raised above the wound surface, irregular, chronically inflamed, contracts poorly due to fibrosis, and is a physical barrier to wound epithelialization (1).

In acute wounds, keratinocytes release pro-inflammatory signals that act as the first alert of skin damage (2,8,11). At the same time, the clotting cascade is activated, and hemostasis is initiated. Platelets release growth factors such as epidermal growth factor (EGF), platelet derived growth factor (PDGF), and transforming growth factor (TGF-beta). Inflammatory cells migrate to the wound site to remove tissue and bacteria. Macrophages initiate the development of granulation tissue and release a variety of cytokines and growth factors. Endothelial cells proliferate and blood vessels form with the stimulation of vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF) and epithelial cell migration and proliferation is stimulated. Finally, once the wound has reached 100% epithelialization, keratinocytes undergo differentiation and stratification and scar remodeling commences (8,11).

Granulation tissue fills wound defects in the early stages, forms a barrier against external contaminants, provides myofibroblasts for wound contraction, and forms the subcutaneous tissue bed over which epithelium can migrate. Ideally, granulation tissue only fills the wound and then contraction and epithelialization occur to complete the healing. When these healing processes are not properly executed or are disrupted, chronic wounds may form. EGT formation is more frequently observed in equine limb than trunk wounds. Horses' non healing wounds are a challenge all equine practitioners face and many times jeopardize a horse's athletic career because of persisting lameness, swollen limbs and extensive scars (1,2,10–16).

According to Maher, 2018 nonhealing wounds in equine patients typically are present in 3 different ways: wounds that do not heal because of local infection, secondary to bone sequestrum, underlying necrotic tendon or ligament, or a residual foreign body that was acquired when the wound occurred. These wounds typically have some degree of EGT. Secondly, wounds that have arrested healing and have become dry. Three chronic cases of EGT on the distal limb that have resulted in large, raised masses. Studies have shown that bandaging or casting equine limb wounds increase the risk of EGT, because of a change in wound oxygen tension to favor angiogenesis and fibroblast proliferation in bandaged wounds and through irritant effects of wound dressings. Wounds with arrested healing processes are dry with minimal to no granulation tissue, and may best be bandaged with a product that stimulates the healing cascade. Silicone gel dressings are highly effective in reducing the tendency of large open limb wounds to develop EGT. Dressings that are fully occlusive seem to occlude microvessels on the wound surface and gradually decrease oxygen tension in the tissues until a point of anoxia, when fibroblasts can no longer function and undergo apoptosis. The ratio of collagen synthesis to degradation is then altered in favor of the latter, thus minimizing fibrosis (1,2,10,17).

The combination of the multifactorial biology of wound healing and the complex pathophysiology of chronic wounds makes them difficult to study. It is also difficult to find an evaluation scale, because there was no agreement on what constitutes a ‘‘good’’ or ‘‘bad’’ scar. The lack of a valid and reliable histomorphologic measure of scarring makes it difficult to compare between various therapies and studies. Singer 2000 and Greenhalgh 1990 scales are histomorphologic scales that incorporate important elements of the skin to describe the quality and quantity of cutaneous scars in human models. Despite the money invested in understanding the biology and pathophysiology of wound healing, in the development of new treatments, and the large number of treatments receiving FDA approval for safety, very few treatments have received FDA approval for efficacy for human chronic wounds treatment since 1997 (18–20).

Tissue bioengineering development is a global concern and different materials are studied and created to be safe, effective and with low cost. NTFS had shown biological potential as a covering for the burning wound in humans due to its collagenous, histological and mechanical similarity to human skin (5,7,21).

Nile Tilapia (*Oreochromis niloticus*) belongs to the Cichlid family and originates from the Nile basin, in Eastern Africa. Its presence is now widespread in tropical and subtropical regions of the world. NTFS is used as a basis for the industry of cosmetics, and the increasing application as a biological dressing material is possible due to easy access, low cost and excellent quality. (5,13,21).

Most of the biomaterials available in Brazil as wound dressings are imported and come at very high cost. NTFS has been suggested as an option of biological material for the management of burns due to characteristics found on this material; the Colony Forming Units found in samples of NTFS before the process of chemical sterilization indicated the presence of normal, non-infectious microbiota, a large composition of type I collagen, morphology similar to human skin and high resistance and tensile extension at break point is founded in this material (21,22). For use in humans, NTFS was subjected to a rigorous process of chemical sterilization, glycerolization and irradiation, followed by microbiological tests for bacteria and fungi, before storage in refrigerated sterile packaging. Prior to its use in the patient, the skin was washed in sterile 0.9% saline for 5 minutes, with this process being repeated three times in a row (6,21). NTFS preparation process showed that the biomaterial did not present variations in its microscopic and tensiometric structure after chemical sterilization and irradiation and recovered its natural consistency after the rehydration process (4,5,21).

So treating chronic wounds is still a challenge for human medicine and also for veterinary. Mainly in equine species, due to EGT in which good results have been recently described on treatments using occlusive curatives. NTFS had a great result as an occlusive curative on humans and mice, making this biomaterial promising for use in horses. (1,2,4,18,23).

2.3 Objective

1. Clinical recovery and weekly macroscopical evaluation of wounds treated with tilapia skin as an occlusive curative on equine wounds during a four weeks treatment. Also to evaluate wound contraction and healing process.

2. Histopathological evaluation of punch biopsy tissue collected every week, in a 28 days period.

3. Evaluation of red blood cells, leukocytes and fibrinogen changes during this study time

2.4 Materials and Methods

2.4.1 Animals

Seven equines and eleven wounds were used during this experiment. The study was case study and prospective. Horses and mules of all ages, breeders and both genders were included.

2.4.2 Wounds Clinical Macroscopical Evaluation

Wounds that could not be resolved by first intention were included, most of them previously treated with different protocols, with unsatisfactory results. Neoplastic wounds were excluded.

Wounds were measured, photographed using one centimeter square scale, cleaned with sterile saline solution 0,9% solution, and punch biopsy tissue was collected for histological diagnosis every week, before a new curative was placed. All wound areas were measured and analyzed on Image J software.

For clinical recovery, macroscopic wound analysis was performed before a new bandage was placed. Color, presence of secretion, smell, crust formation, moisture of the wound and any other alteration information were collected in the patient's medical record weekly during bandage change protocol.

Contraction area evaluation was calculated using Greenhalgh, 1990 formula: D % Closed = [(Area on Day 0- Open Area on Final Day)/Area on Day 0] X 100. The formula was also used to calculate weekly contraction; using the week evaluated as Open Area on Final Day and the week before Area on Day 0, for example D14 % Closed = [(Area on Day 7- Area on Day 14)/Area on Day 7] X 100.

2.4.3 Local

During the four week-treatment horses were housed in Equine Hospital of Faculty of Veterinary Medicine and Animal Science USP, Mounted Police of São Paulo and UNIP Campina`s large animals` hospital.

2.4.4 Clinical Laboratory Samples

EDTA tube was used for blood samples, collected by jugular puncture weekly, on the same day the bandages were changed. This procedure was done for four weeks (28 days). Red blood cells, leukocytes and fibrinogen were analyzed.

2.4.5 Bandage changing protocol

Wounds were cleaned with sterile saline solution 0,9% . NTFS was washed with sterile saline solution 0,9% three times for 15 minutes. NTFS was fixed around the wound with instantanel glue (Superbonder®), the side with scales facing outside (Figure 5). A bandage was fixed over NTFS to keep it moisturized. On the trunk, sterile gauze and adhesive tape was used. On the limbs gauze, roll cotton and self bonding material (Vet wrap®) were used. Same tension was used during the vet wrap application to keep it in place and not cause wound damage. Equine Wound Management was used as a guidance for bandages confection (Eggleston, 2018). Repellent Silver spray was applied around the bandage.

On day zero (D0) wounds were photographed using one centimeter square scale, cleaned with sterile saline solution 0,9%, and punch biopsy tissue was collected for histological diagnosis. NTFS was applied for the first time using the bandage changing protocol. The same protocol was repeated on day 7 (D7), day 14 (D14), day 21 (D21) and day 28 (D28) of study period (Figure 1).

Animal 5 LHL wound: (A) with tilapia skin occlusive bandage, (B) during curative change, using a one cm square scale for picturing wound and measuring wound area on Image J program.

Figure 1 - Equine wound treated with Nile tilapia fish skin: curative change and measuring takes process

Source: Cicolo, 2022

A pilot study was performed in horses 1 and 2, in which NTFS from a farm market was used (25). The NTFS was embedded in chlorhexidine 2% for 20 min and left in the freezer for a maximum of 3 months as described (25). The other animals were treated with tilapia skin provided by Dr Edmar Maciel teams. These NTFS went through chlorhexidine baths, microbiology tests, UV radiation and are kept in glycerol (6,21).

Tilapia skin has a non pathogenic microbiota, also equine chronic wounds are contaminated, so we assume that there’s no significant difference between both

NTFS used, since both of than go throw chlorhexidine protocols before being used (1,6,11).

2.4.6 Histological evaluation

Biopsies were collected every week during bandage change from the wound edge. A 6.0 mm punch biopsy was used. Lidocaine 20% was used for local blocking before biopsy and, when necessary, xylazine (0,5 to 1,0 mg/ Kg) was used as a sedative.

Each week, the biopsy was collected from a different part of the wound, following a clockwise pattern. A total of five tissue biopsies were taken from each wound. Biopsy tissue fragments were kept in formol 10% solution. A 0.6 mm punch biopsy tissue was collected for histological diagnosis on day zero.

The fragments were sliced in vertical orientation, photographed and included on a paraphyne block. The block was sliced, prepared in a glass microscope slide and stained with hematoxylin-eosin (H&E).

Histological analysis was done by Prof ª Drª Lilian Rose Marques de Sá in the Pathology Department of the Faculty of Veterinary Medicine and Animal Science USP.

Singer, 2000 burne scale and Greenhalgh, 1990 scale, both human chronic wound healing scales, were used for histomorphological during day 0, 7, 14, 21 and 28 (19,20).

2.4.7 - Statistical analysis

For the statistical analysis, initially, a descriptive analysis of the data was carried out with an estimate of mean, median, standard deviation, 25% and 75% percentile of the variables Leukocyte, Fibrinogen, Red blood cells and wound area reduction. To assess the differences between the moments, first the quantitative variables were tested for normal distribution with the Shapiro-wilk normality test to determine the parametric and non-parametric approach. For variables with normal distribution, the difference between the moments was verified with the ANOVA test of repeated measures. For variables without normal distribution, the difference between the moments was verified with the Friedman test. For better visualization of these analyses, bar graphs and boxplots were produced. All tests were considered significant when $p<0.05$ and the analyzes were performed in the R 4.0.4 environment (24).

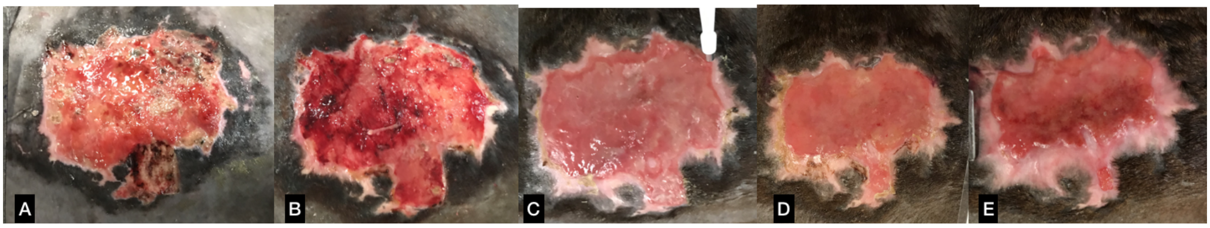
2.5 Results

2.5.1 Wounds Clinical Macroscopical Evaluation

During the study period no allergic reaction to tilapia skin was noticed. In most wounds an irregular, red, coarse surface of exuberant granulation tissue changed to pink, with smooth surface and epithelized edges of a healthy cicatrization tissue after two weeks. In the second week, yellow secretion was observed in all wounds. The healing process of each subject was particular, as detailed below.

Animal 1.

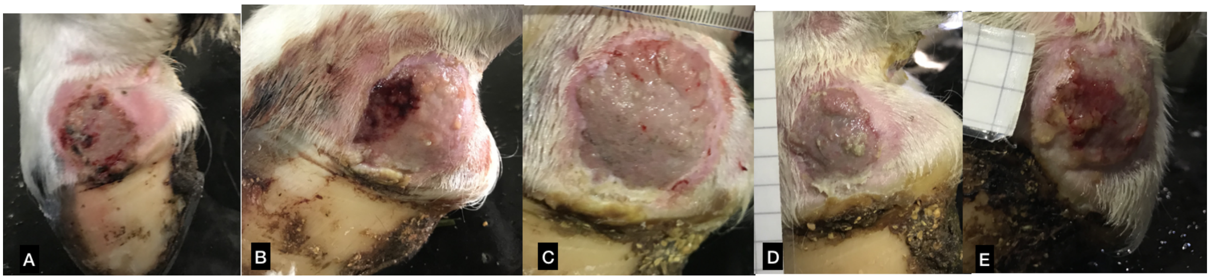
Male, eight years old, Mangalarga Marchador. Presented wounds on back, LHL, lateral aspect near proximal interphalangeal joint, RHL, lateral aspect near proximal interphalangeal joint, on the LFL, also proximal to interphalangeal joint. The wounds had been treated with commercial equine topical ointment, with poor results. Histological diagnosis was of eosinophilic inflammation and habronema, treated with oral ivermectin on day 0. The wounds showed good healing in the beginning, but due to itching, the horse bit the wound repeatedly, causing delay in the healing process. During the first two weeks there was an excessive, dense and yellow secretion (Figure 5), mainly in the back wound, as described in Table 2. Tilapia skin suffered some degradation during the first weeks, when there was presence of intense secretion. After this period there was less secretion and wounds changed from red appearance to pink, it was possible to see an epithelialization in the wound edges. There was also a reduction in wound size (Figure 2, 3, 4 and Table 1). After 28 days the subject was treated with prometazina to aid the itching aspect.



Animal 1 back wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 2 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 1 back wound

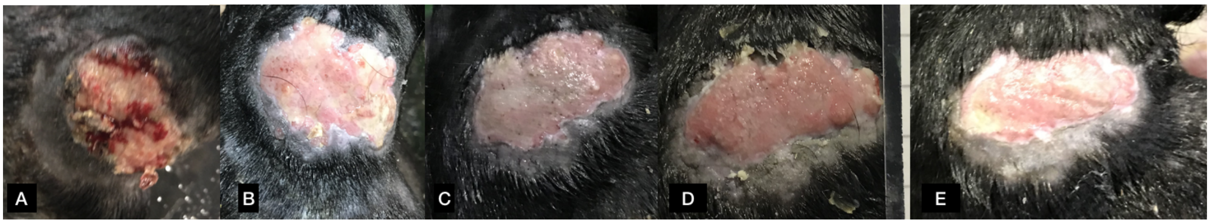
Source: Cicolo, 2022



Animal 1 LFL wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 3 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 1 left forward limb

Source: Cicolo, 2022



Animal 1 RHL wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 4 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 1 right hind limb

Source: Cicolo, 2022

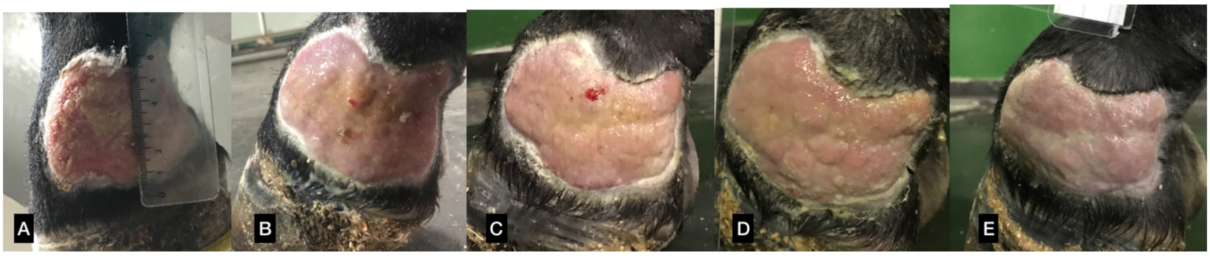
Yellow secretion described on macroscopical wound treated with Nile tilapia fish skin occlusive curative, during bandage changing.

Figure 5 - Yellow secretion formed on bandage of equine wound treated with Nile tilapia fish skin occlusive curative

Source: Cicolo, 2022

Animal 2.

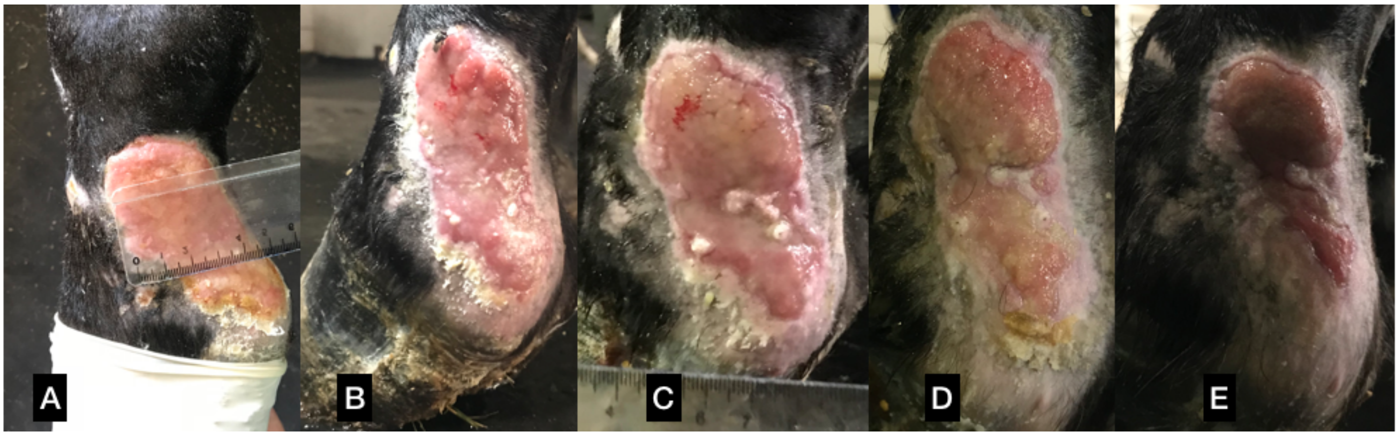
Male, twelve years old, Mangalarga Marchador. Presented wound on LHL and LFL, both lateral aspects, between interphalangeal joint and metacarpophalangeal joint. Histological diagnosis was habronema and treated with oral ivermectin on day 0. Two surgical procedures had been done to remove exuberant granulation tissue with poor results. During the first and second weeks of tilapia treatment, the wound showed a lot of secretions and a strong smell. Bandages had to be changed after 3 days. The tilapia skin used as curative suffered degradation during these weeks, with intense secretion. After two weeks granulation tissue changed from red to soft pink and from irregular to regular with a scarred edge. Wound area showed a reduction (Figure 6 and 7; Table 1), it was possible to observe epitization in the wound board after 4 weeks treatment.



(A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 6 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 2 animal 2 left hind limb

Source: Cicolo, 2022



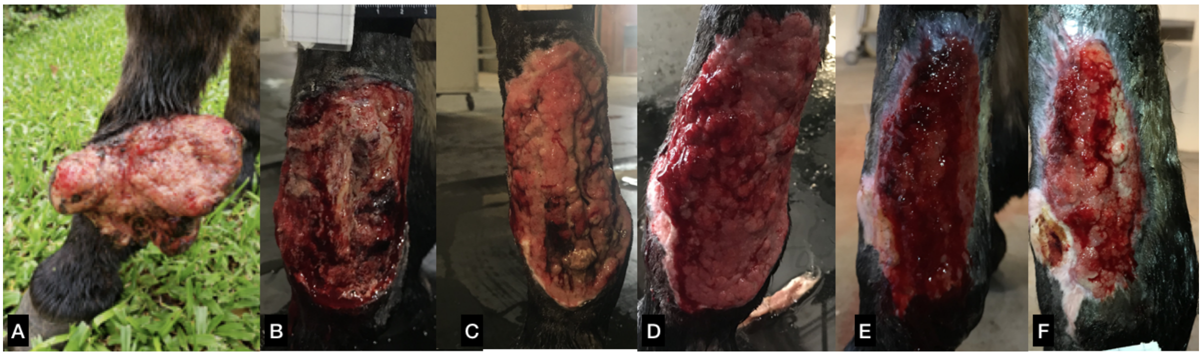
Animal 2 LFL wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 7 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 2 left forward limb

Source: Cicolo, 2022

Animal 3.

Female, 15 years old, mule. Presented LFL wound, lateral, dorsal and medial aspect. Historical EGT surgical removal one day before. Histological diagnosis was habronema and it was treated with ivermectin and surgery for excision of granulation tissue one day before tilapia skin treatment started. The cicatrization process was much faster than animal 1 and 2 (Table 1 and Figure 8), but had also a lot of secretion in the two first weeks. Intense degradation of tilapia skin was not observed in this case. It was noticed a remarkable wound cicatrization during the 4 week period.



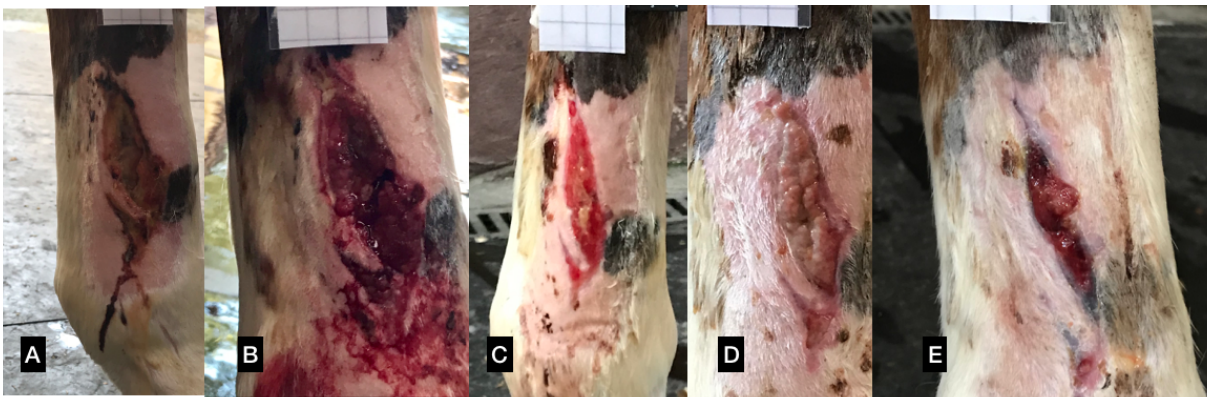
Animal 3 LFL wound: (A) before surgical excision, (B) before applying Nile tilapia fish skin occlusive curative , (C) after 7 days, (D) after 14 days, (E) after 21 days, (F) 28 after days.

Figure 8 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 3 left forward limb

Source: Cicolo, 2022

Animal 4.

Male, seven year old, Brazilian Warmblood. Presented with a small wound in LHL, on the medial aspect of the left metatarso. A traumatic injury that had been treated for 2 weeks with daily wash and topic comercial ointment, with poor results. In the first two weeks there was presence of secretion and tilapia skin partially disintegrated, after which the skin developed pink rather than red color, and good wound resolution was observed. After five weeks with tilapia skin treatment the wound healed completely. (Table 1, Figure 9).



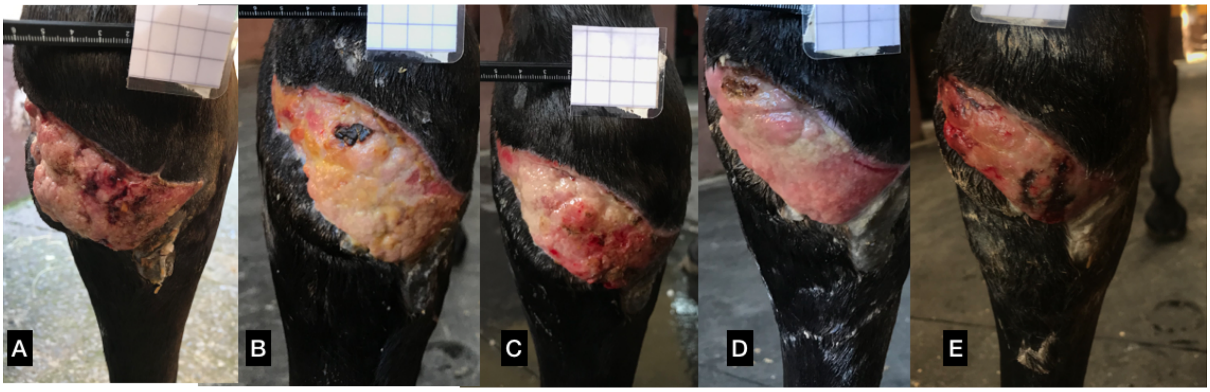
Animal 4 LHL wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 9 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 4 left hind limb

Source: Cicolo, 2022

Animal 5.

Female, five year old, Brazilian Warmblood. Show jumper presented with a suture breakdown in an injury at the LHL, plantar aspect of tarsal joint. After applying tilapia skin, the wound had a great amount of secretion, but due to small size it was not necessary to change bandages before one week. After the first week great improvement was observed, granulation tissue had completely disappeared and tissue had changed from red to healthy pink color (Figure 10 and Table 1). During treatment there was remodeling of fibrotic tissue in the subcutaneous area around the wound, which improved joint mobility during four weeks and after wound healing. The affected hook had a very similar appearance to the health joint, which represents a great result for an athletic horse. The horse went back training and performed in 1,20 m classes.



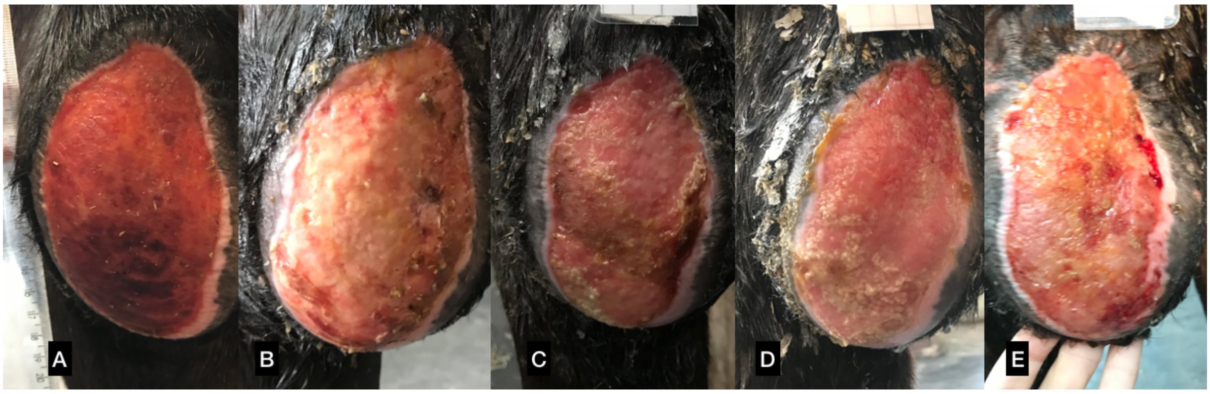
Animal 5 LHL wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 10 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 5 left hind limb

Source: Cicolo, 2022

Animal 6.

Male, 4 years old, Brazilian equestrian breed. Presented with a wound LFL, palmar aspect of the olecranon area, with sutures breakdown. It has been treated with moxibustion for a few weeks, with poor results. The horse was treated for five weeks with tilapia skin, with a decrease in the inflammatory process and reduction of fibrotic tissue around the wound. Cicatrization tissue changed from red to a smooth pink color, but it was opted for surgical resolution due to difficulty keeping the bandage in place during lay down periods (Table 1 Figure 11).



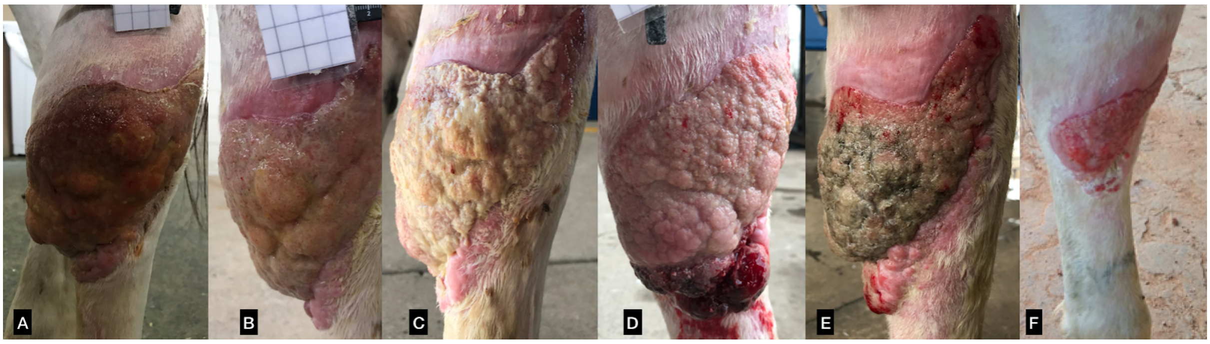
Animal 6 LFL wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 11 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 6 left forward limb

Source: Cicolo, 2022

Animal 7.

Female, seven year old, mixed breed. Presented with a large wound at LHL, lateral, dorsal and medial aspect of the tarsal joint. Surgical removal of exuberant granulation tissue was performed twice, with no success in tissue granulation control (Figure 12). Treatment with tilapia skin showed secretion and degradation of material during the first three weeks, followed by great result in wound healing (Table 1). After that period, granulation tissue couldn't be controlled and had slow resolution. After 28 days it was decided to use copper sulfate for 10 minutes on the granulation tissue, rinsing with sterile saline solution 0,9% solution, application of tilapia skin and bandaging. With this method a faster result was observed and the wound healed in three months.

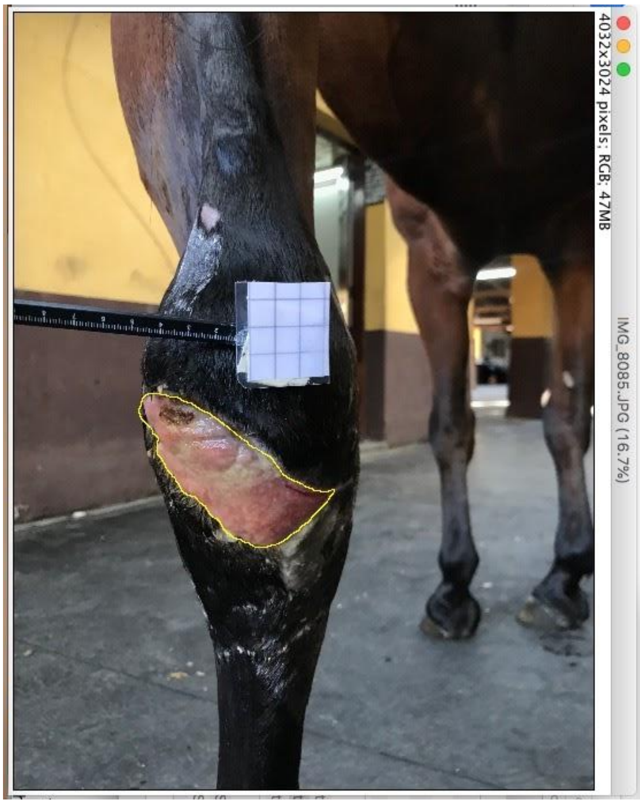


Animal 7 LHL wound: (A) before applying Nile tilapia fish skin occlusive curative , (B) after 7 days, (C) after 14 days, (D) after 21 days, (E) 28 after days.

Figure 12 - Nile tilapia fish skin occlusive curative used in equine wound day 0 to 28: animal 7 left hind limb

Source: Cicolo, 2022

Wounds of all animals were photographed using one centimeter square scale on days: D0, D7, D14, D21 and D28 (Figure 13). All wound areas were calculated using Image J software and were described on table 2.



Calculating area, using one centimeter square scale on Image J program, of wound treated with Nile tilapia fish skin as an occlusive curative in equine.

Figure 13 - Equine wound treated with Nile tilapia fish skin as occlusive curative measuring area procedure

Source: Cicolo, 2022

Table 1 - Equine wound treated with Nile tilapia fish skin as occlusive curative during 28 days macroscopic evaluation and wound measure

Date	Day	Animal	Wound location	Area (cm²)	Width (cm)	Height (cm)	Macroscopic evaluation of the wound
22/11/2018	0	1	Back	110,40	13,00	12,00	Red color, presence of crust, necrotic yellow and black areas. Bleeding areas
29/11/2018	7	1	Back	56,90	10,00	8,90	Dark pink color, yellow secretion and epithelized edges. Foul smell noticed. Tilapia skin was degraded.
07/12/2018	14	1	Back	51,50	9,30	8,20	Pink color, yellow secretion and epithelized edges. Presence of hair in epithelialized area. Foul smell noticed
14/12/2018	21	1	Back	43,70	8,70	8,20	Light pink color, less yellow secretion and epithelized edges. Presence of hair in epithelialized area
21/12/2018	28	1	Back	38,40	8,70	5,60	Pink color, white and epithelized edges. Presence of hair in epithelialized area

22/11/2018	0	1	RHL	-	-	-	Coarse exuberant granulation tissue, passing edges, red color, bleeding, presence of crust and black spots
29/11/2018	7	1	RHL	9,10	4,00	2,60	Smooth granulation tissue, no trespassing edges, pink color, yellow secretion and epithelized edges. Skin was degraded.
07/12/2018	14	1	RHL	8,90	4,00	2,40	Pink color, epithelized edges, yellow secretion
14/12/2018	21	1	RHL	6,10	4,00	2,00	Pink color, epithelized edges, yellow secretion and a few exuberant granulation tissue in the middle
21/12/2018	28	1	RHL	5,00	3,90	1,50	Light pink color, white and epithelized edges
22/11/2018	0	1	LHL	-	-	-	Red color, bleeding, presence of crust and black spots
29/11/2018	7	1	LHL	12,16	3,30	4,70	Smooth granulation tissue, no trespassing edges, pink color, yellow secretion and epithelized edges. Skin was degraded.
07/12/2018	14	1	LHL	12,00	3,20	3,90	Smooth granulation tissue trespassing edges, pink color, less yellow secretion and epithelized edges
14/12/2018	21	1	LHL	7,20	3,00	3,10	Pink color, smooth tissue. Less yellow secretion
21/12/2018	28	1	LHL	7,00	3,00	3,00	Pink color, smooth tissue, reddish spots (bitted). Less yellow secretion
22/11/2018	0	1	LFL	13,50	3,50	3,10	Pink color, exuberant granulation tissue, bleedin
29/11/2018	7	1	LFL	8,30	3,50	2,70	Pink color, exuberant granulation tissue, bleeding, better aspect than week before. Skin was degraded.
07/12/2018	14	1	LFL	3,00	2,70	1,70	Pink color, smooth tissu
14/12/2018	21	1	LFL	2,50	2,60	1,50	Pink color, smooth and epithelized edges
21/12/2018	28	1	LFL	2,30	2,30	1,40	Dark pink color, smooth, granulation tissue in the middle and epithelized edges
23/11/2018	0	2	LHL	31,80	6,70	6,20	Dark pink color, granulation tissue trespassing edges, edges not well defined (after surgical excision
29/11/2018	7	2	LHL	29,30	6,60	4,50	Pink color, smooth, white and epithelized edges, intense yellow secretion. Foul smell noticed.Skin was degraded.
07/12/2018	14	2	LHL	26,30	6,50	4,40	Pink color, smooth, white and epithelized edges, intense yellow secretion. Granulation tissue in the middle. Foul smell noticed
14/12/2018	21	2	LHL	25,20	6,40	4,00	Pink color, smooth, white and epithelized edge
21/12/2018	28	2	LHL	23,00	5,70	3,70	Light pink color, smooth, white and epithelized edge
23/11/2018	0	2	LFL	25,20	4,30	8,20	Dark pink and yellow color, granulation tissue trespassing edges, edges not well defined (after surgical excision
29/11/2018	7	2	LFL	19,40	3,10	7,80	Smooth granulation tissue, no trespassing edges, pink color, yellow secretion and epithelized edges. Foul smell noticed. Skin was degraded.

07/12/2018	14	2	LFL	12,80	2,80	6,10	Pink color, smooth, intense yellow secretion, epithelized edges. Foul smell noticed
14/12/2018	21	2	LFL	12,20	2,50	6,70	Pink color, smooth, epithelized edges. Dorsal edge presenting exuberant granulation tissue. It seems this area has lost contact with tilapia curative
21/12/2018	28	2	LFL	7,80	2,40	5,40	Light pink, extensive area healed. Smooth tissue trespassing edges. Dark pink color on the dorsal edge
27/02/2019	0	3	LFL	70,00	12,00	13,00	Extensive yellow and whitish wound, bleeding on LFL, metatarsus latera,dorsal and medial aspects. Presence of cloth and fibrotic tissue.
07/03/2019	7	3	LFL	60,76	12,00	6,05	Friable tilapia skin around. Foul smell. Edges showing contraction and healing. Pink tissue, bleeding. Necrotic and fibrotic spots. Intense yellow secretion
14/03/2019	14	3	LFL	49,08	12,00	5,50	Pink color, healing edges, cranial edge showing pink granulation tissue. Bleeding in middle area. Less yellow secretion and still a foul smell, but a little better than the week before
21/03/2019	21	3	LFL	42,30	11,70	4,20	Pink color and smooth tissue color, withesh edges showing contraction.a. Bleed when curative was removed since tilapia skin was adhered to the wound. Less yellow secretion
28/03/2019	28	3	LFL	26,00	8,68	4,00	Tilapia skin was adhered to the wound. Less yellow secretion, dark pink / red color, exuberant granulation tissue. Bleeding in the middle. Healing, contraction, white and epithelized edges.
02/07/2019	0	4	LHL	12,40	2,80	8,60	Traumatic deep wound in Left high limb (LHL) in metatarsus medial face. Yellow with purulent secretion
10/07/2019	7	4	LHL	11,90	2,80	7,90	pink color, coarse surface in the whole wound. Bad smell noticed. Skin was degraded.
16/07/2019	14	4	LHL	7,30	1,50	7,80	Pink red color, smooth surface and contraction
23/07/2019	21	4	LHL	6,80	1,40	7,80	Tilapia skin disintegrates (dust). Pink color, slightly green, coarse surface and contraction
30/07/2019	28	4	LHL	3,10	0,70	4,40	Red color, smooth surface, lot of contraction wound
02/07/2019	0	5	LRL	36,00	10,50	6,00	Wound in dorsal face of left hind limb (LHL), pink exuberant granulation tissue, dark spots (hematoma)
10/07/2019	7	5	LRL	32,00	9,50	6,00	Light pink, lot yellow secretion, dark, smooth area in the middle, tissue not trespassing edges. Skin was degraded.
16/07/2019	14	5	LRL	25,00	9,20	5,80	Pink color, red dots, lot of yellow secretion and a yellow jello recovering wound Retraction of wound and epithelization of caudal and medial edge

23/07/2019	21	5	LRL	25,00	9,10	5,20	Pink color, little bit darker on dorsal side (crust), smooth surface, trespassing edge. Retraction of medial, dorsal and lateral edge. coarse grain on medial size. yellow secretion and foul smell
30/07/2019	28	5	LRL	23,10	8,90	5,50	Took off a bandage one day before. Pink color, reddish spots and crust. Contraction of lateral, medial and dorsal edge. Less secretion. Foul smell
11/07/2019	0	6	LFL	62,46	7,10	11,70	LFL wound, palmas aspect, near to proximal ulna spot. Sutures breakdown, treated with mocha. Red color wound, withesh edges
16/07/2019	7	6	LFL	43,49	5,70	9,70	Took off a bandage, was changed once within 3 days. Pink color, yellow secretion, yellow jello recovering. Bad smell. Decrease in swelling around the wound
23/07/2019	14	6	LFL	36,00	5,20	8,70	Bandage changed twice in a week. Pink color, less secretion. Animal had a bite wound. Foul smell noticed
30/07/2019	21	6	LFL	60,00	2,00	10,00	Bandage changed twice in a week. Pink color, yellow crust. Animal had bitten the wound. Foul smell noticed
06/08/2019	28	6	LFL	44,70	5,30	10,00	Changed bandage once before in the week. Dark pink, reddish color, yellow crust, contraction
08/11/02019	0	7	LHL	103,00	16,24	9,28	Large wound at the lateral, dorsal and medial aspect of the hock on LHL. Exuberant granulation tissue, trespassing edges. Red and yellow color. Nodular formation, il-defined edges
13/11/02019	7	7	LHL	75,30	14,30	9,00	Coarse surface, trespassing edges. Define edges, yellow color, density yellow jell recovering wound. Bad smell noticed.Skin was degraded.
21/11/02019	14	7	LHL	72,00	11,60	8,70	Coarse surface, trespassing edges. Define edges, yellow color, density yellow jell recovering wound. Edges contraction. Foul smell noticed
27/11/02019	21	7	LHL	68,50	9,80	8,50	Pink color, whitie coarse surface, ventral area bleeding (animal had bitten it), well defined edges, epithelization and yellow secretion
04/12/02019	28	7	LHL	66,00	9,00	8,00	Coarse surfain, yellow - green color, yellow secretion, well defined edges, retraction of wound edges

Macroscopic Evaluation and Wound Measure off all Seven Animals, evaluated day 0 to 28, animal number 1 to 7, Limb location of the wound, total area, width, height of wound and a macroscopical wound evaluation including color, aspect of tissue, smell, secretion, crust, bleeding and any other wound microscopically observed.
Source: Cicolo, 2022

Table 2 - Equine wound treated with Nile tilapia fish skin during 28 days period weekly percentagem closed wound and total wound percentage closed

D %	Animal 1 Back	Animal 1 RHL	Animal 1 LHL	Animal 1 LFL	Animal 2 LHL	Animal 2 LFL	Animal 3	Animal 4	Animal 5	Animal 6	Animal 7	Weekly Regression Average	% Weekly Regression
D 7%	1,941			1,627	1,085	1,299	1,152	1,042	1,125	1,436	1,368	1,342	34%
D 14%	1,105	1,022	1,014	2,767	1,114	1,516	1,238	1,630	1,280	1,208	1,046	1,358	35%
D 21%	1,178	1,459	1,667	1,200	1,044	1,049	1,159	1,074	1,000	0,600	1,051	1,135	13%
D 28%	1,138	1,220	1,029	1,087	1,096	1,564	1,628	2,194	1,082	1,342	1,038	1,311	31%
D % Closed	65,24%	59,04%	57,11%	82,96%	27,67%	69,04%	62,85%	75,00%	35,83%	28,43%	35,92%	1,286	

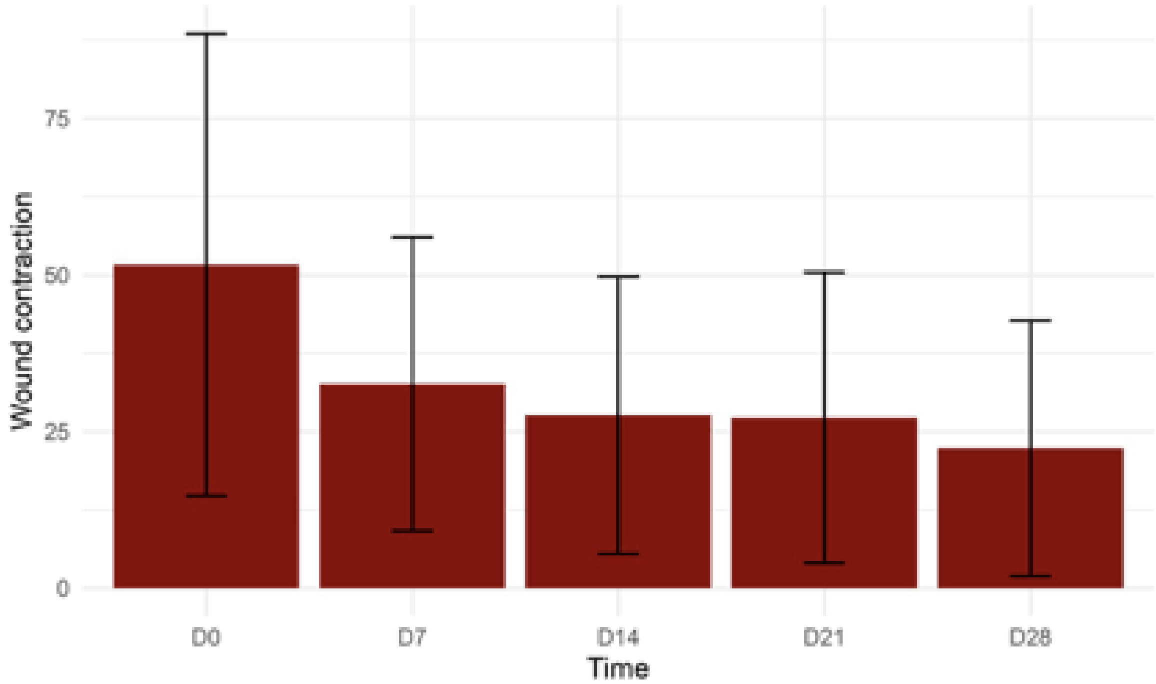
Contraction area evaluation was calculated using Greenhalgh, 1990 formula: D % Closed = [(Area on Day 0- Open Area on Final Day)/Area on Day 0] X 100. The formula was also used to calculate weekly contraction; using the week evaluated as Open Area on Final Day and the week before Area on Day 0, for example D14 % Closed = [(Area on Day 7- Area on Day 14)/Area on Day 7] X 100.For measuring wound percentage close to animal 1 RHR and LHL we used the mean regression of all other wounds to extrapolate D0 wounds, because it was not taken.
Source: Cicolo, 2022

Table 3 - Equine wound treated with Nile tilapia fish skin occlusive curative analysis of area variable separated by the five moments of interest and also the comparison between them

VAriable	M	MD	DP	IIQ	M	MD	DP	IIQ	M	MD	DP	IIQ	M	MD	DP	IIQ	M	MD	DP	IIQ	p-value*
Wound Area	51,64	36	36,88	44,8	32,58	29,3	23,45	38,15	27,62	25	22,16	32,05	27,23	25	23,21	36	22,33	23	20,41	26,2	<0,001

*Friedman Test

There was a statistically significant difference between the moments (p=<0.001), and the differences occurred between the moments D0 x D7 (p=0.039), D0 x D14 (p=0.039), D0 x D28 (p=0.039), D7 x D14 (p=0.01), D7 x D28 (p=0.02) and D21 x D28 (p=0.38).
Source: Cicolo, 2022



Bar graph for the mean and standard deviation of the equine wound treated with nile tilapia fish skin area variable at each moment

Figure 14 - Equine wounds treated with Nile tilapia fish skin curative occlusive area changes during 28 days period

Source: Cicolo, 2022

There was a very significant statistical difference in wound contraction results p=<0,001(Table - 3 and Figure 14). Wound area was also used for percentage closed calculation (Table - 2), that was quantified weekly and final (D% closed). The weekly wound percentage close was 28% of totally wound regression close. An individual difference was observed between wounds; 27,67% on animal 2 LHL and the biggest on was animal 1 LFL 82,96%.

Weekly wound regression close were 34%, 35%, 13% and 31% of total wound regression close, the lowest percentage was in the third week. A great healing percentage was observed in the first and second week. Same results were seen on a Friedman test (Figure 14) D7 (p=0.039), D14 (p=0.039), D28 (p=0.039), D14 (p=0.01), D28 (p=0.38) and D7 x D28 (p=0.02) .

2.5.2 Macroscopical evaluation of biopsy tissue

Punch biopsy from equine wound treated with Nile tilapia fish skin occlusive curative from wound edge

Figure 15 - Equine wound treated with Nile tilapia fish skin occlusive curative punch biopsy macroscopical healing process evaluation

Source: Cicolo, 2022

During punch biopsy tissue analysis, (Figure 15) it was observed that in all animals at the first two weeks (D0 and D7) punch biopsy showed friable, pink and easy bleeding tissue during collection. From the third week onwards (D14, 21 and 28) the punch tissue was whiter, presenting fibrous consistency.

2.5.3 Histopathological evaluation

Morphological diagnosis and description were performed for all tissues collected by punch biopsies (Figure 16). Histopathological description was performed for all animals during day 0, 7, 14, 21 and 28. A qualitative morphological evaluation of all animals, for each period, was done.

Punch biopsy tissue procedure has been performed on the wound edge.

Figure 16 - Equine wound treated with Nile tilapia fish skin occlusive curative punch biopsy on the wound edge

Source: Cicolo, 2022

Histological diagnosis was taken using day zero tissue from punch biopsy. Three animals presented habronema diagnostic, one animal presented drug reaction diagnostic and 3 animals presented EGT (Figure 17).

Animal	Early Morphological Diagnosis (Day 0)	Final Morphological Diagnosis (Day 28)
1	Habronemose	Repair Process
2	Habronemose	Repair Process
3	Habronemose	Repair Process
4	Drug Reaction	Repair Process with granulation tissue
5	Exuberant granulation tissue	Exuberant granulation tissue
6	Exuberant granulation tissue	Exuberant granulation tissue
7	Exuberant granulation tissue	Exuberant granulation tissue

Morphological diagnosis by animal (one to seven) on Day 0 and Day 28.

Figure 17 - Morphological diagnosis of wounds by animal equine wound treated with Nile tilapia fish skin occlusive curative morphological diagnosis of wounds by animal

Source: Cicolo, 2022

Singer 2000 scale and Greenhalgh 1990 were used to score histopathological results. Both showed a final score lower than initial score (Figure 18 and 19). Qualitative histological evaluation of all wounds showed exuberant granulation tissue in the first week (Figure 20). In the first two weeks, intense yellow secretion was observed macroscopically, and histological analysis showed an important neutrophilic infiltrate.

Day	Animal 1 (back)	Animal 1 (RHL)	Animal 1 (LHL)	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6	Animal 7	Average
0	-	-	-	4	-	-	5	3	1	3,2
7	6	-	-	0	3	10	3	4	1	3,8
14	1	0	1	1	2	4	1	4	2	1,7
21	1		2	1	2	4	2	4	1	2,1
28	3	1	4	2	1	5	2	5	1	2,6

Singer 2000 Scale evaluation of tilapia skin occlusive curative on equine wound, points scored by animal x time on Singer 2000 scale

Figure 18 - Singer 2000 scale evaluation equine wound treated with Nile tilapia fish skin occlusive curative

Source: Cicolo, 2022

Day	Animal 1 (back)	Animal 1 (RHL)	Animal 1 (LHL)	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6	Animal 7	Average
0	-	-	-	10	-	-	10	7	10	9,7
7	8	-	1	5	4	10	10	10	10	7,2
14	8	-	4	8	8	4	10	10	6	7,2
21	7	8	9	10	8	4	10	10	7	8,1
28	7	1	10	7	10	1	10	10	9	7,2

Greenhalgh 1990 Scale evaluation of tilapia skin occlusive curative on equine wound, points scored by animal x time on Greenhalgh 1990 scale

Figure 19 - Greenhalgh 1990 scale evaluation equine wound treated with Nile tilapia fish skin occlusive curative

Source: Cicolo, 2022

Day	Animal 1	Animal 2	Animal 3	Animal 4	Animal 5	Animal 6	Animal 7
0		0	0	0	0	0	1
7	0	1	1	1	1	1	1
14	1	1	1	1	1	1	1
21	1	1	1	1	1	1	1
28	1	1	1	1	1	1	1

Presence of neutrophilic infiltrate on wounds by animal, according to the evaluated day (0,7,14,21 and 28), being zero absence and one presence

Figure 20 - Neutrophilic infiltrate equine wound treated with Nile tilapia fish skin occlusive curative

Source: Cicolo, 2022

Qualitative histological evaluation of all wounds showed exuberant granulation tissue in the first week (Figure 21). In the first two weeks, intense yellow secretion was observed macroscopically, and histological analysis showed an important neutrophilic infiltrate.

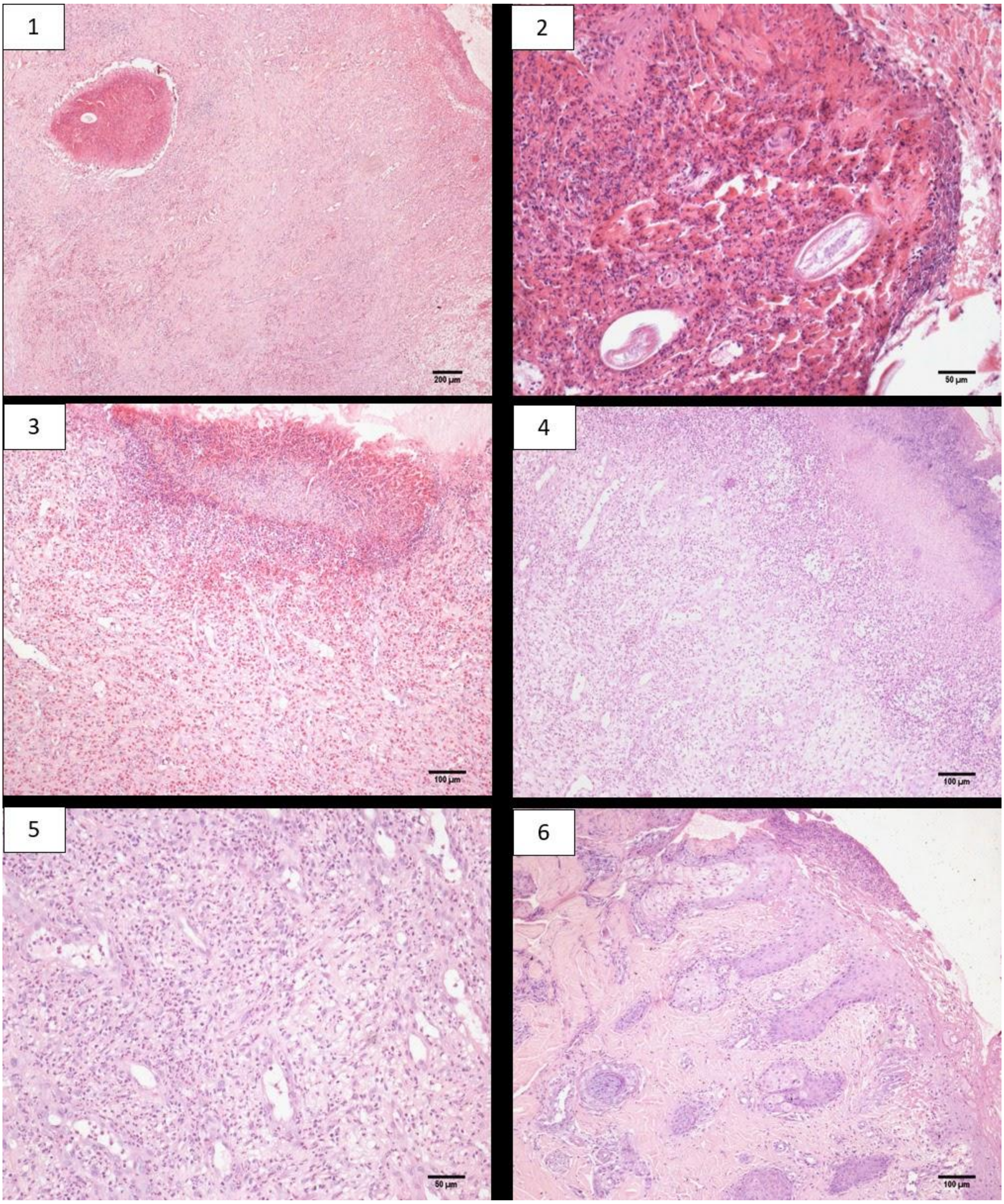
Day	Qualitative morphological evaluation
0	All animals presented ulcerated wounds. Animals 1, 2 and 3 presented an eosinophilic inflammatory process. 2 and 3 had eosinophilic granulomas with larvae. Animal 6 had neovascularization and necrosis. Animal 4,5 and 7 had granulation tissue
7	All animals presented ulcerated wounds, and the predominant inflammatory process was neutrophilic in 1, 3 and 4. Animals 5 and 7 continued to show granulation tissue. Animal 3 showed necrosis and thrombosis. In animals 6 and 4, the tissue repair process begins to be observed. Animal 1 showed edema and neovascularization
14	Animal 2 did not have an ulcerated wound and all the others did. Animals 1, 3 and 4 had a neutrophilic process. Patient 1 had an eosinophilic process and 2 had a lymphocytic inflammatory process. Animals 1 and 2 had fibroplasia and neovascularization and 3 and 4 had tissue repair, and 5, 6 and 7 had granulation tissue.
21	Animal 1 had no ulcer and all the others did. Animals 1, 2 and 3 had a neutrophilic inflammatory process. Animal 2 had an eosinophilic inflammatory process, in addition to a neutrophilic one. Animal 1 had histiocytic inflammatory infiltrate. (1 = pyogranulomatous 2 = neutrophilic and eosinophilic). Patient 1 and 2 had neovascularization. 1,5,6 and 7 had granulation tissue. Animal 2 had fibroplasia. Patient 3 and 4 tissue repair
28	All had an ulcerated wound. Animals 1, 2 and 3 had neutrophilic inflammatory infiltrate. Animals 1 and 2 with eosinophilic infiltrate. Patient 1 lymphocytic and histiocytic infiltrate. (1 = mixed infiltrate). 1 and 2 had neovascularization and fibroplasia, 3 had tissue repair. Animal 2 edema. Patient 5, 6 and 7 granulation tissue

Qualitative morphological evaluation of all animals (one to seven) described by day, being evaluated days 0, 7, 14, 21 and 28, in the respective lines.

Figure 21 - Morphological evaluation equine wound treated with Nile tilapia fish skin occlusive curative

Source: Cicolo, 2022

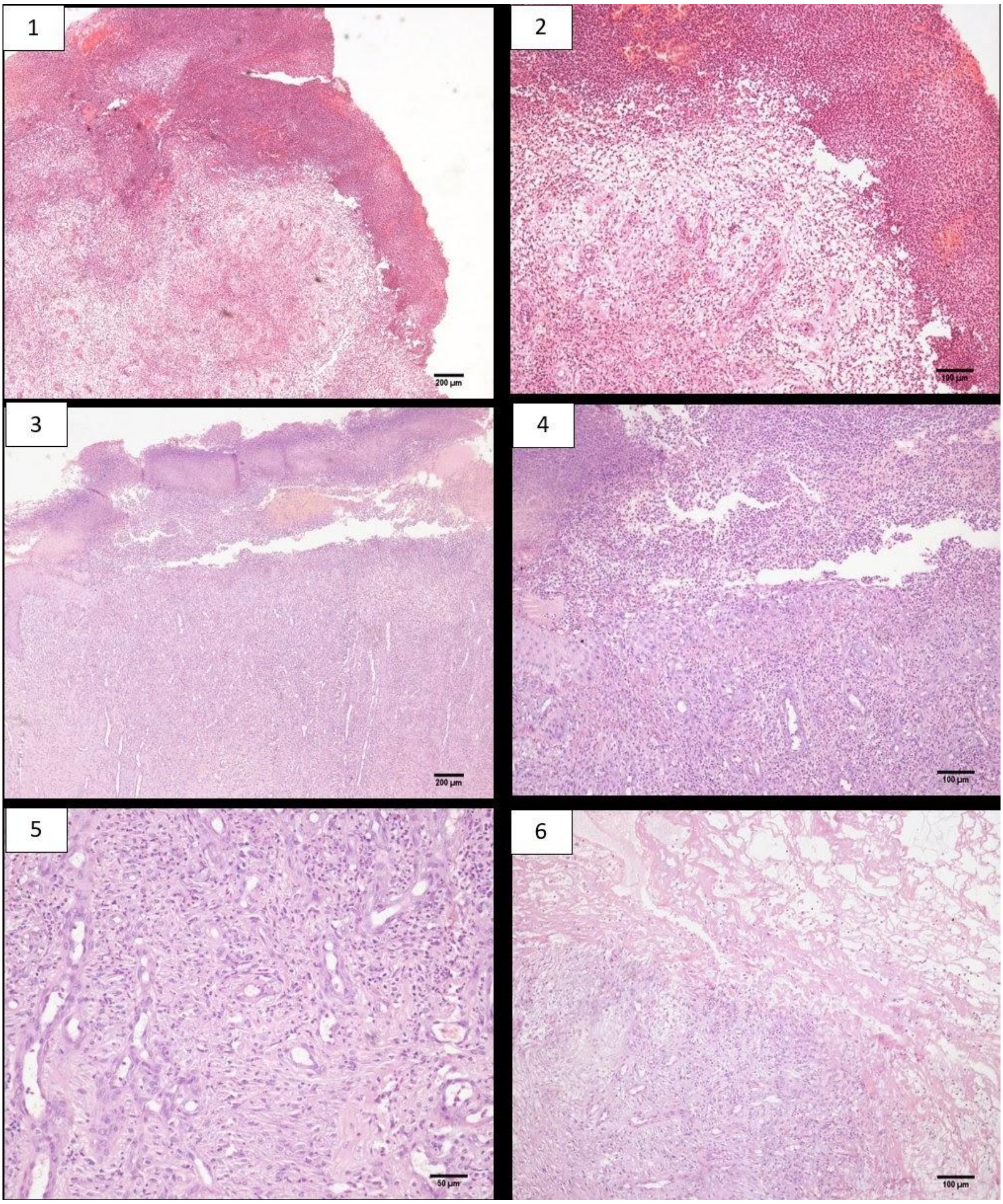
Based on histopathological analyses (Figure - 22 to 31) NTFS induces an acute inflammatory response (AIR) with a lot of exudate, consequently the healing process is directed to granulation and healing. AIR persisted until day 21, even if fibroplasia and neovascularization occurs on deep dermis since day seven. In habronema cases eosinophils were observed until day 14, but fewer in number than neutrophils.



(1) Animal 2 LHL wound. Skin, eosinophilic exudative and necrotic ulcerative dermatitis. Eosinophilic granuloma formation cross-section of degenerate larva (arrow). Habronema. (2) Animal 1 LFL wound. Skin, eosinophilic granuloma formation cross-section of intact larva (arrow) Habronema. (3) Animal 1 back wound. Skin, eosinophilic exudative crust and ulcerative dermatitis. (4) Animal 5 LHL wound. Skin, ulceration, exudation and organizing granulation tissue. EGT (5) Animal 5 LHL wound. Skin, granulation tissue (6) Animal 4 LHL wound. Skin, ulceration associated to neutrophilic exudation, trauma in healing process. Hematoxylin and eosin.

Figure 22 - Photomicrograph of equine chronic wounds treated with Nile tilapia fish skin occlusive curative on day zero

Source: Cicolo, 2022

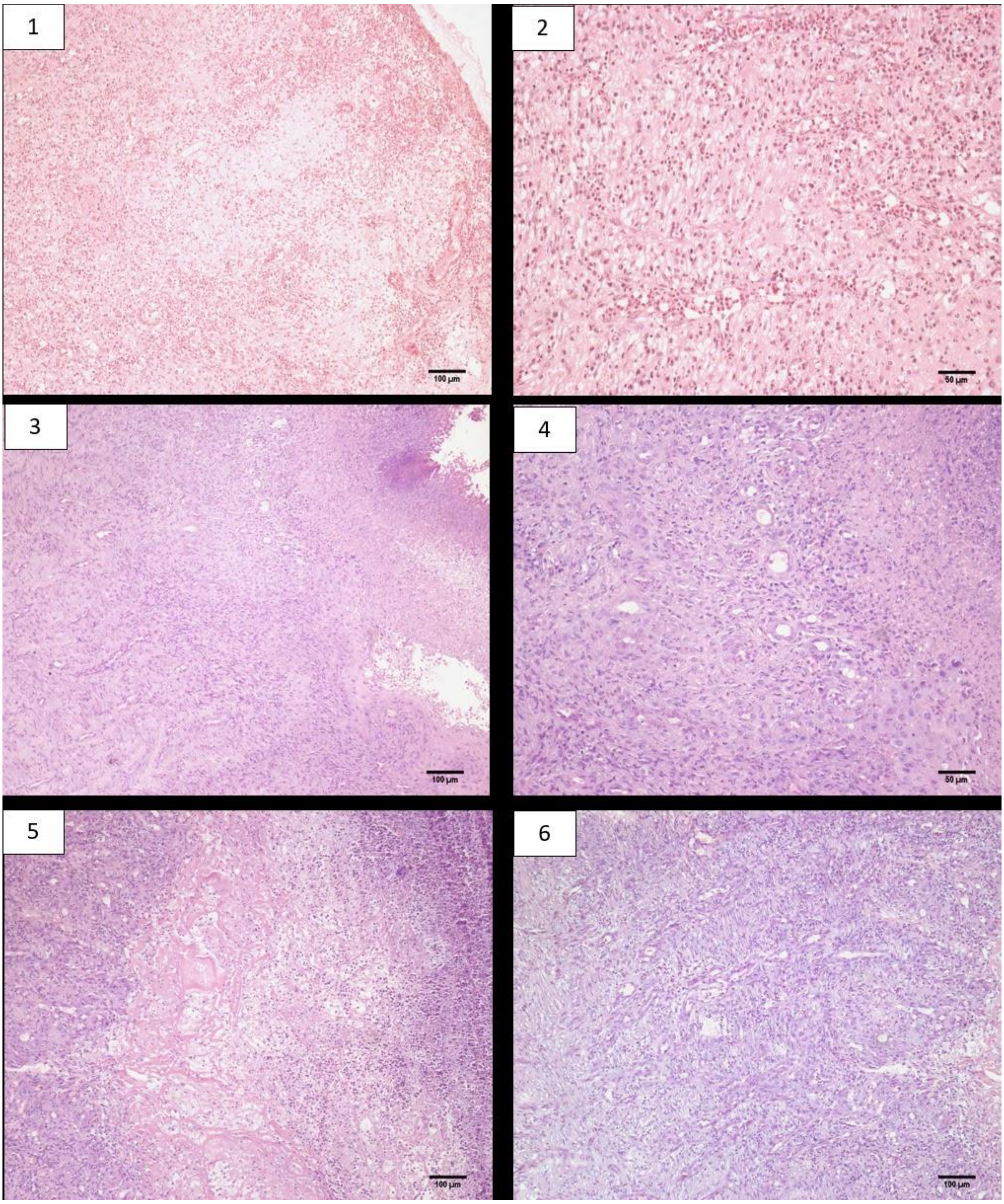


(1) Animal 2 LHL wound. Skin, neutrophilic crust (arrow) exudative process and ulcerative dermatitis. (2) Animal 1 LFL wound. Skin, neutrophilic crust (arrow) exudative process and ulcerative dermatitis (3) Animal 6 LFL wound. Skin, ulceration and neutrophilic exudative (arrow) in organizing granulation tissue. (4) Animal 5 LHL wound. Skin, ulceration associated to neutrophilic exudation (arrow), organizing granulation tissue (5) Animal 5

LHL wound. Skin, granulation tissue. Vessels (arrow) perpendicular to the collagen bundle (6) Animal 4 LHL wound. Skin, ulceration, neutrophilic fibrin exudation (arrow). Hematoxylin and eosin.

Figure 23 - Photomicrograph of equine chronic wounds treated with Nile tilapia fish skin occlusive curative on day seven

Source: Cicolo, 2022

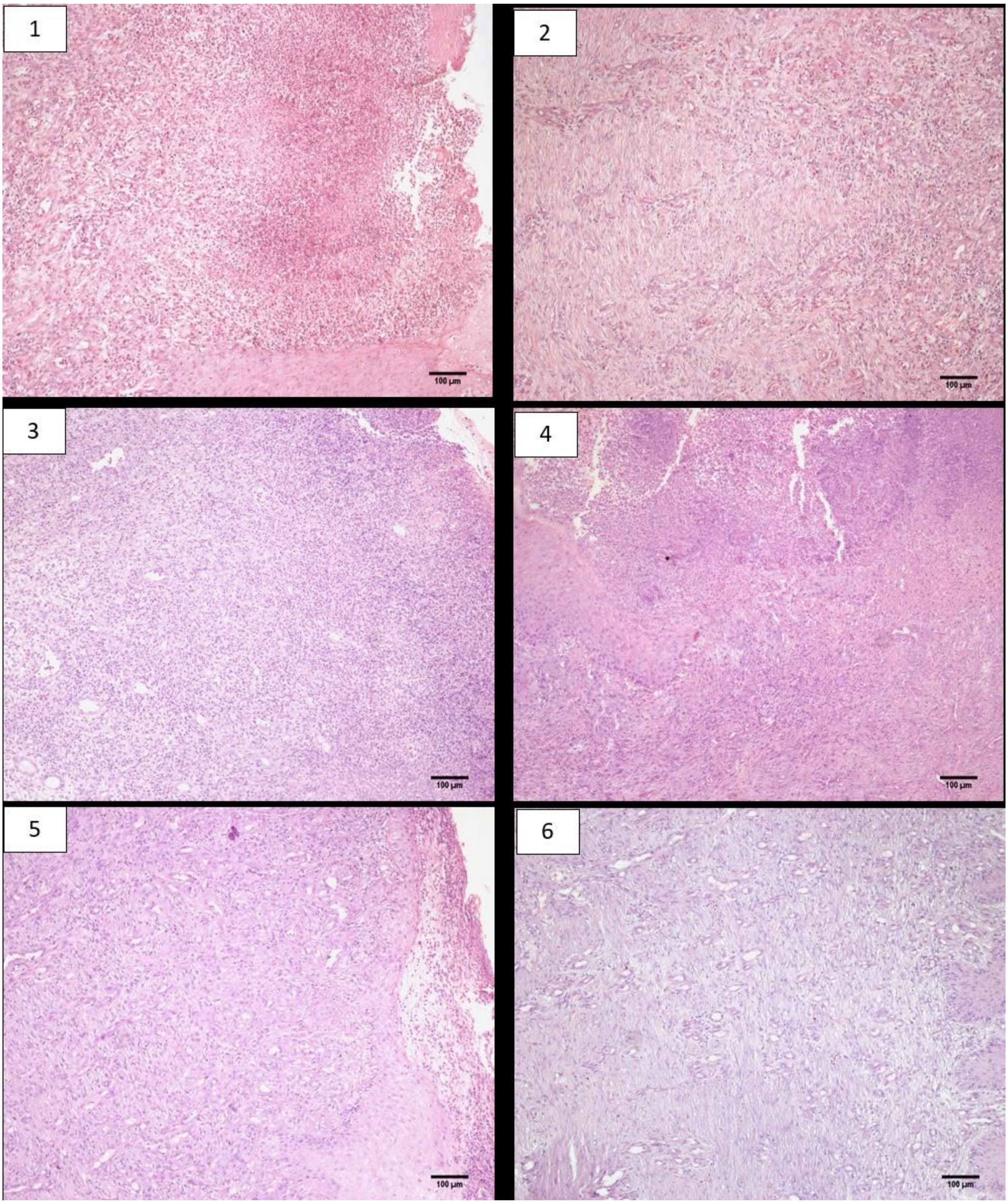


(1) Animal 3 LFL wound. Skin, granulation tissue and superficial neutrophilic exudation (arrow). (2) Animal 3 LFL wound. Skin, granulation tissue, vessels (arrow) perpendicular to the collagen bundle (3) Animal 6 LFL wound.

Skin, neutrophilic exudation (arrow) in organizing granulation tissue. (4) Animal 5 LHL wound. Skin, neutrophilic exudation (arrow) in organizing granulation tissue. (5) Animal 4 LHL wound. Skin, granulation tissue. neutrophilic fibrin exudation (arrow). (6) Animal 4 LHL wound. Skin, granulation tissue, vessels (arrow) perpendicular to the collagen bundle. Hematoxylin and eosin.

Figure 24 - Photomicrograph of equine chronic wounds treated with Nile tilapia fish skin occlusive curative on day 14

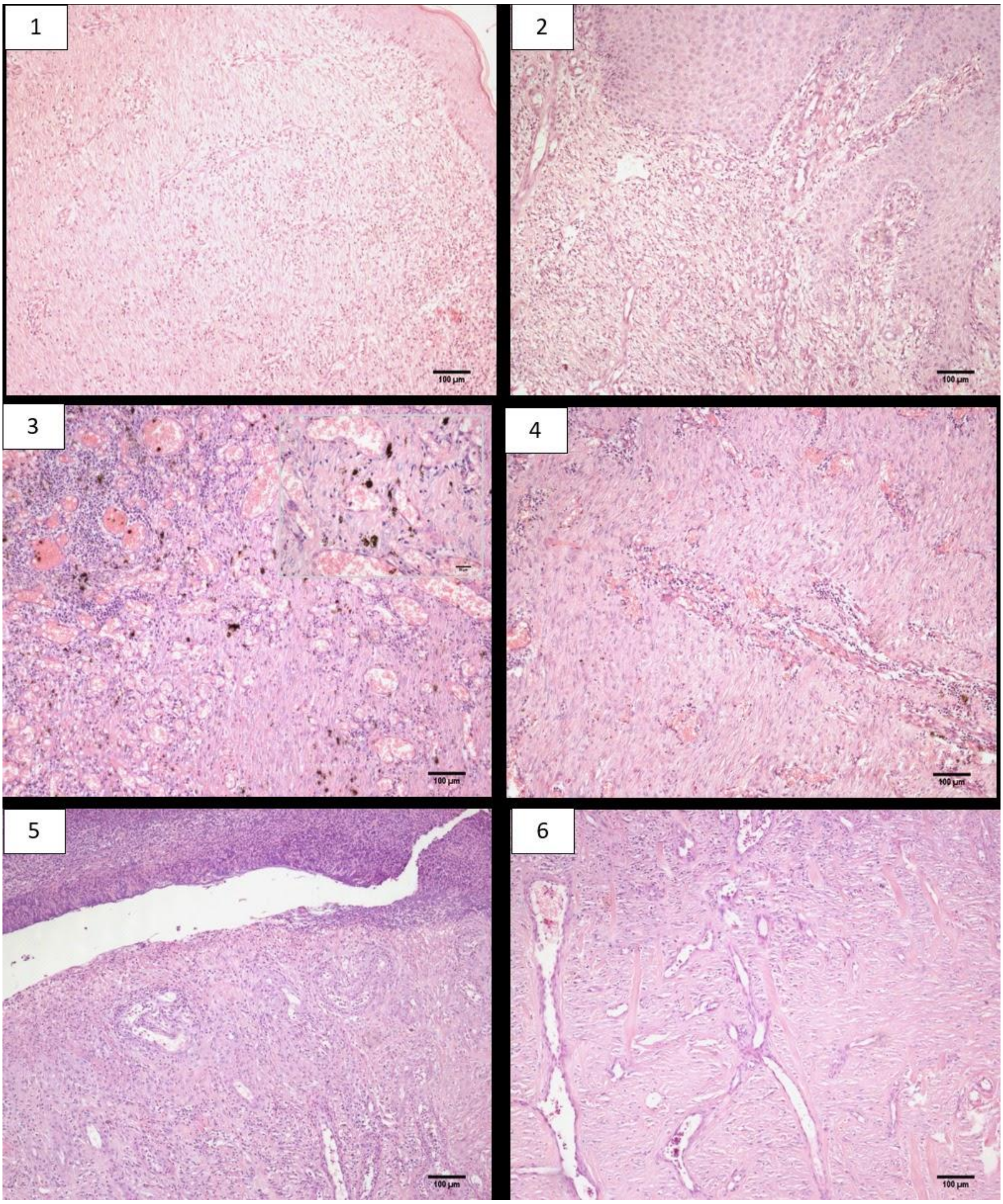
Source: Cicolo, 2022



(1) Animal 1 wound. Skin, granulation tissue and superficial neutrophilic exudation (arrow). Hyperplasia on epidermis (arrow head). (2) Animal 1 wound. Skin, granulation tissue, vessels perpendicular to the collagen bundle and neutrophils (arrow) (3) Animal 5 LHL wound. Skin, neutrophilic exudation (arrow) in organizing granulation tissue. (4) Animal 6 LFL wound. Skin, neutrophilic exudation (arrow) in organizing granulation tissue and re-epithelialization tissue (arrow head). (5) Animal 4 LHL wound. Skin, granulation tissue. Neutrophilic exudation (arrow) and re-epithelialization tissue (6) Animal 4 LHL wound. Skin, healing granulation tissue, vessels (arrow) perpendicular to the collagen bundle. Hematoxylin and eosin.

Figure 25 - Photomicrograph of equine chronic wounds treated with Nile tilapia fish skin occlusive curative on day 21

Source: Cicolo, 2022



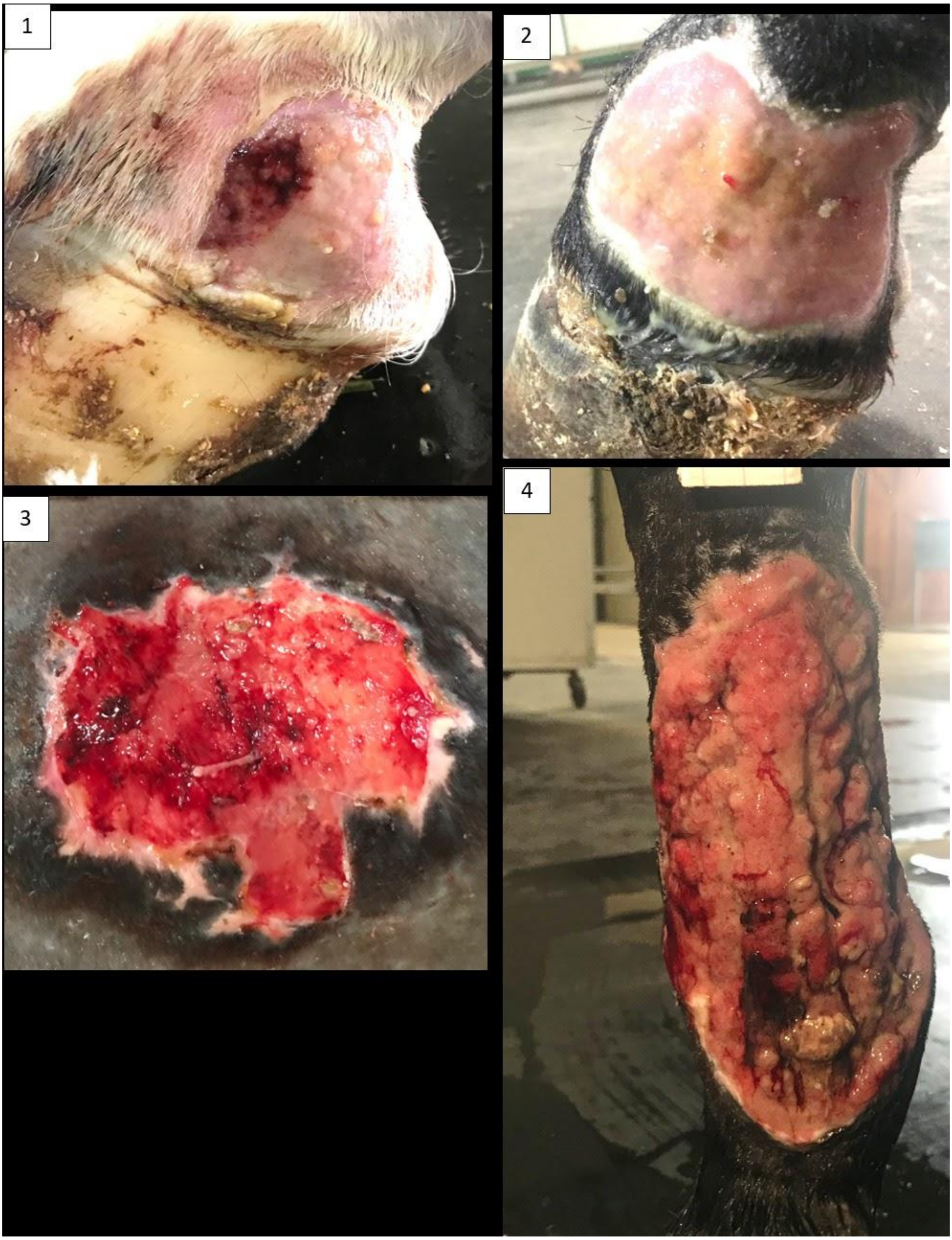
(1) Animal 1 wound. Skin, re-epithelialized epidermis and maturing granulation tissue. Hyperplasia on epidermis (arrow head). (2) Animal 2 wound. Skin, re-epithelialized and hyperplastic epidermis. Maturing granulation tissue (3) Animal 3 LFL wound. Skin, maturing granulation tissue. neutrophilic and histiocytic infiltrate in dermis, presenting dark pigments. (4) Animal 3 LFL wound. Skin, Maturing granulation tissue. (5) Animal 6 LFL wound. Skin, granulation tissue. Neutrophilic exudation (arrow) and re-epithelialization tissue (6) Animal 6 LFL wound. Skin, maturing granulation tissue, vessels (arrow) perpendicular to the collagen bundle. Hematoxylin and eosin.

Figure 26 - Photomicrograph of equine chronic wounds treated with Nile tilapia fish skin occlusive curative on day 28

Source: Cicolo, 2022

Equine Habronema Wound 0 (1) Animal 1 LFL (2) Animal 2 LHL (3) Animal 1 back wound (4) Animal 3 LFL wound

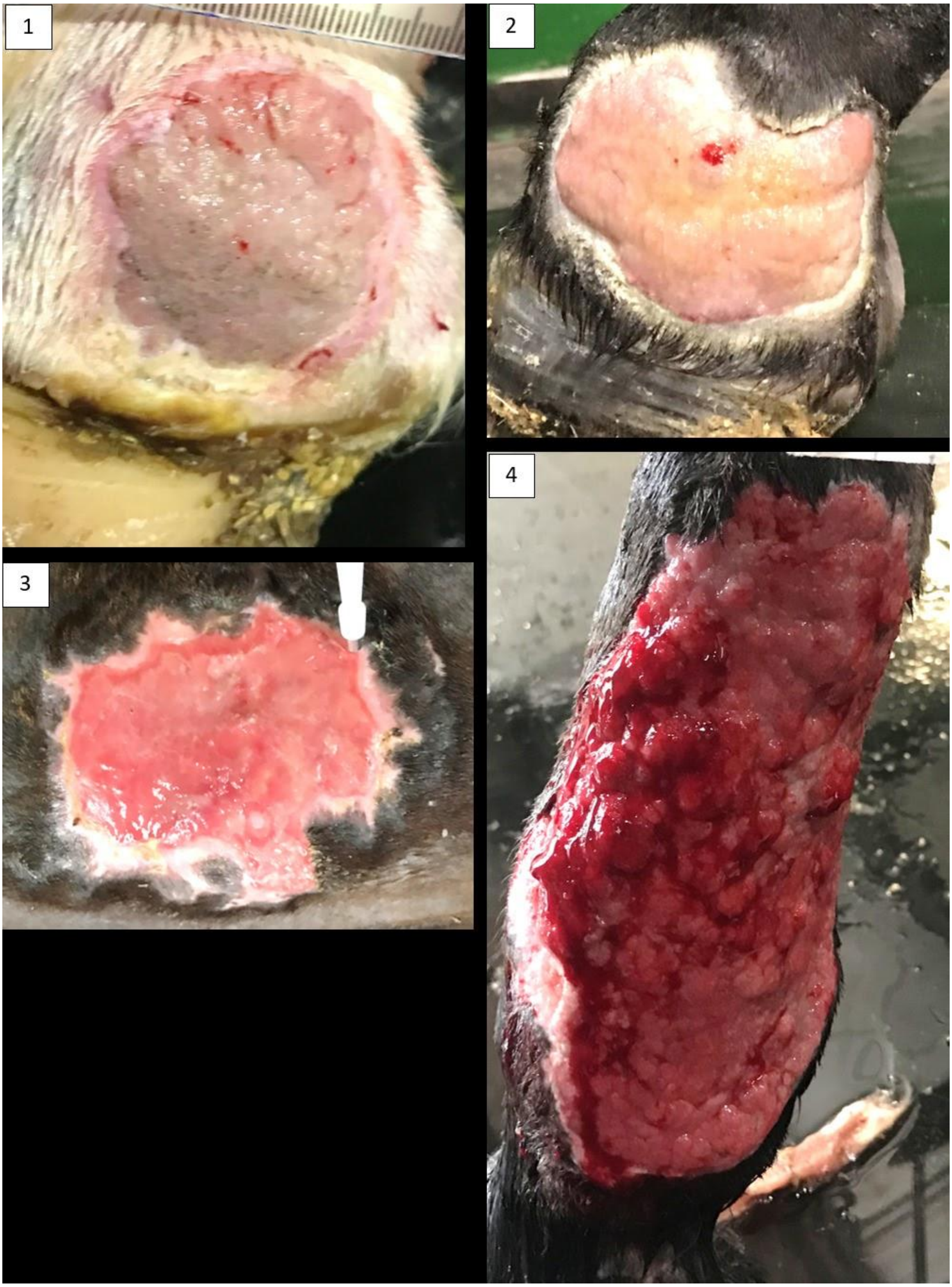
Figure 27 - Chronic skin lesions due to habronema in the limb and back of horses treated with Nile tilapia fish skin occlusive curative, day zero



Source: Cicolo, 2022
Equine Habronema Wound (1) Animal 1 LFL (2) Animal 2 LHL (3) Animal 1 back wound (4) Animal 3 LFL wound

Figure 28 - Chronic skin lesions due to habronema in the limb and back of horses treated with Nile tilapia fish skin occlusive curative, day 7

Source: Cicolo, 2022



Equine Habronema Wound (1) Animal 1 LFL (2) Animal 2 LHL (3) Animal 1 back wound (4) Animal 3 LFL wound

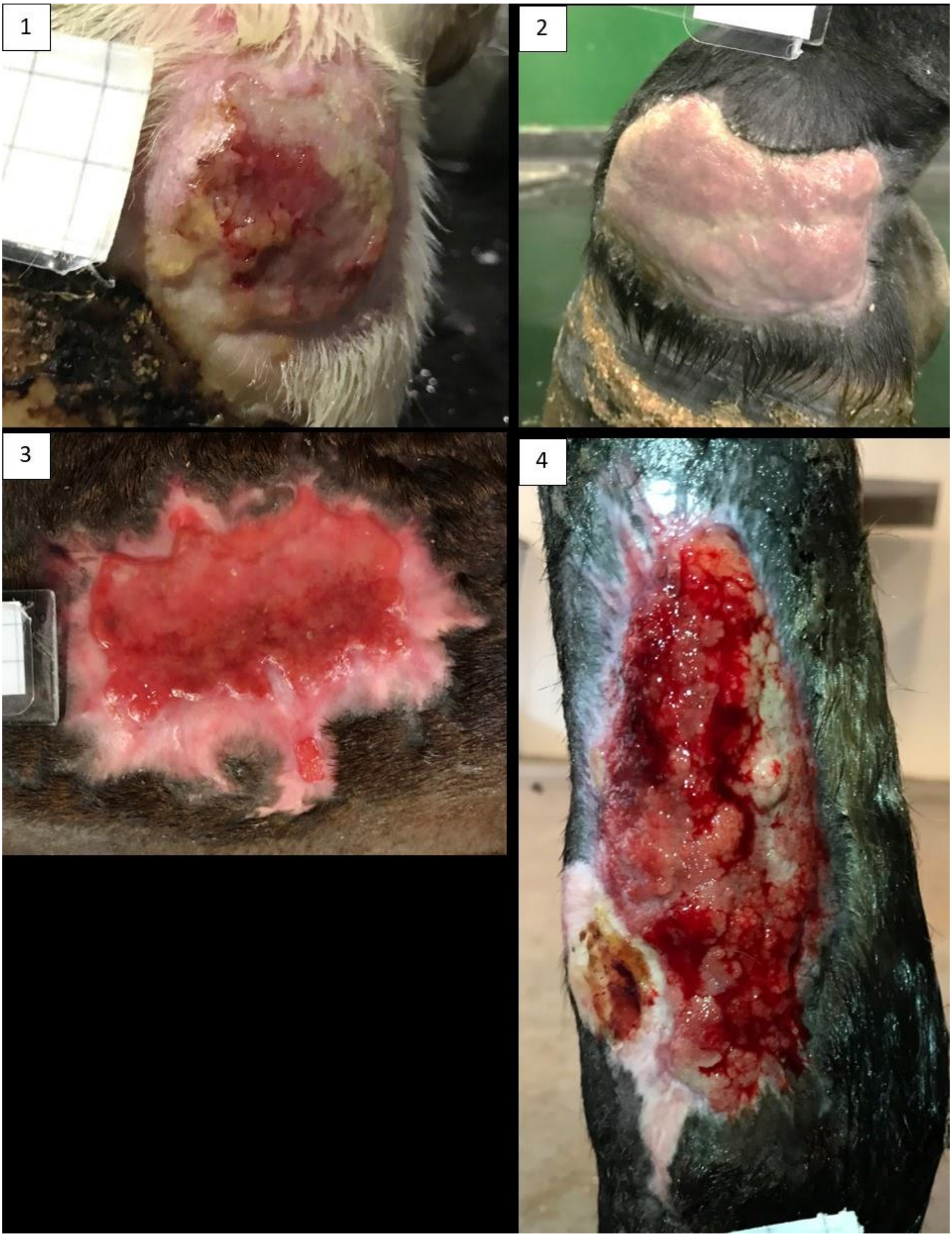
Figure 29 - Chronic skin lesions due to habronema in the limb and back of horses treated with Nile tilapia fish skin occlusive curative, day 14

Source: Cicolo, 2022

Equine Habronema Wound (1) Animal 1 LFL (2) Animal 2 LHL (3) Animal 1 back wound (4) Animal 3 LFL wound

Figure 30 - Chronic skin lesions due to habronema in the limb and back of horses treated with Nile tilapia fish skin occlusive curative, day 21

Source: Cicolo, 2022



Equine Habronema Wound (1) Animal 1 LFL (2) Animal 2 LHL (3) Animal 1 back wound (4) Animal 3 LFL wound

Figure 31 - Macroscopical view of chronicles habronema equine wound treated with Nile tilapia fish skin occlusive curative on day 28

Source: Cicolo, 2022

2.5.4 Clinical Laboratory analysis result

Descriptive and inferential statistical analysis of blood red cell, leukocytes and fibrinogen were done.

Table 4 - Clinical laboratory variables equine wound treated with Nile tilapia fish occlusive curative skin

Variable	N	M	MD	Min	Max	DP	1Q	3Q	IIQ	p-value*
Leukocytes	33	9,14	9,1	5,8	13,3	1,79	7,84	10,1	2,26	0,82
Fibrinogen	33	324,45	246	161	678	148,36	200	400	200	<0,001
Red Cells	33	7,55	7,3	5,6	9,7	1,31	6,4	8,9	2,5	0,0054
* Shapiro-Wilk Test										

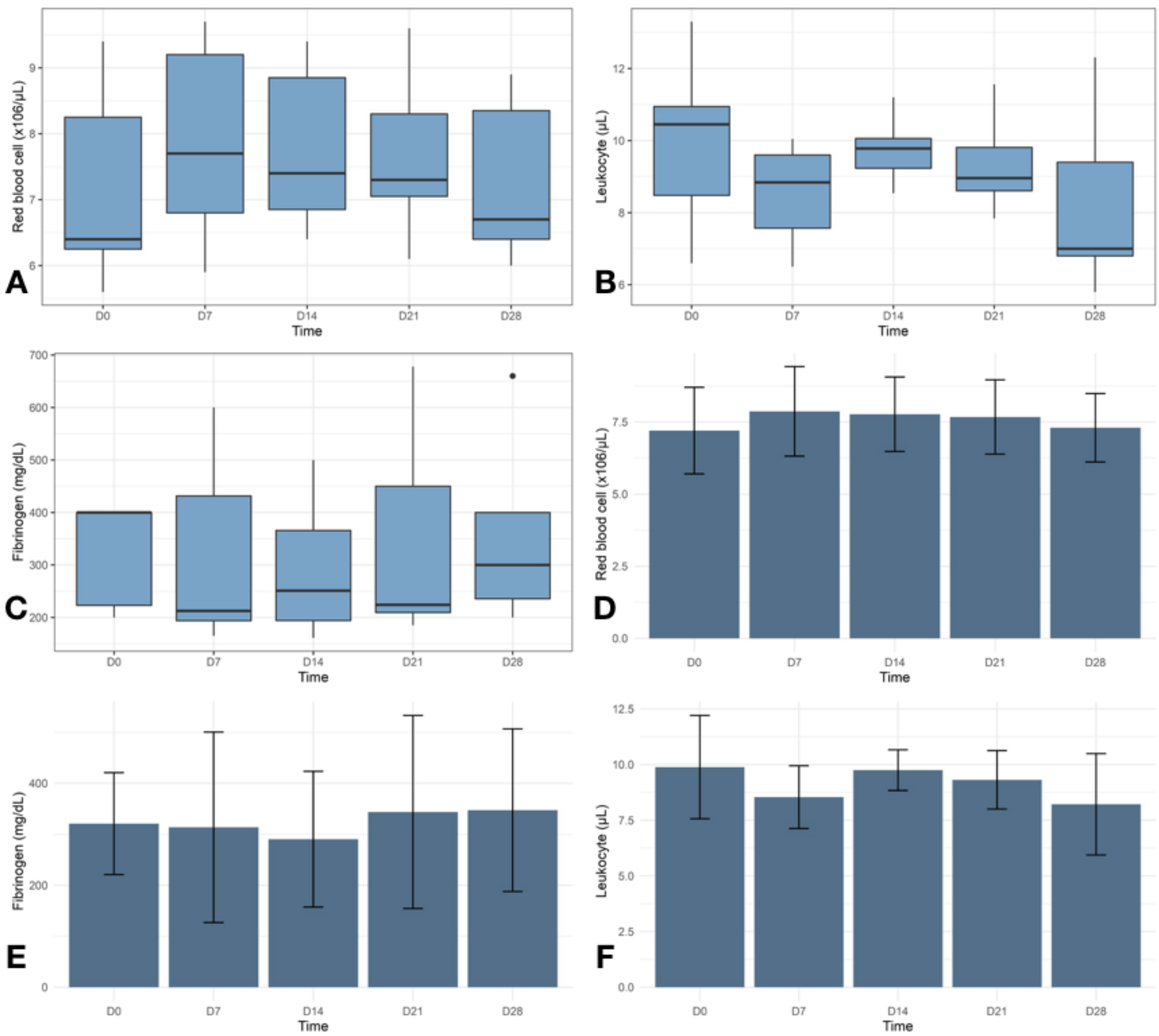
Source: Cicolo, 2022

Table 5 - Comparison of the clinical laboratory variables equine wound treated with Nile tilapia fish skin occlusive curative

Variable	M		DP	IIQ	M	MD	DP	IIQ	M	MD	DP	IIQ	M	MD	DP	IIQ	M	MD	DP	IIQ	p-value*
Leukocytes	9,89	10,45	2,32	2,47	8,54	8,84	1,41	2,03	9,75	9,78	0,91	0,83	9,31	8,96	1,31	1,2	8,22	7,00	2,28	2,6	0,386
Fibrinogen	320,86	400	99,89	177	313,67	212,5	186,79	237,75	290,33	251	133,03	171,75	343,71	224	189,33	240,5	347,29	300	159,4	164,5	0,689
Red Cells	7,20	6,4	1,50	2	7,87	7,7	1,55	2,4	7,77	7,4	1,29	2	7,67	7,3	1,29	1,25	7,3	6,7	1,19	1,95	0,241

Source: Cicolo, 2022

Shapiro-Wilk Test shows the descriptive analysis of the variables separated by the five moments of interest and also the comparison between them. There was no statistically significant difference between the moments for the variables (Table 4-5 and Figure - 35).



Boxplot: (A) of the red blood cell variable at each time point, (B) leukocytes variable, (C) fibrinogen variable; Bar graph for the mean and standard deviation of the (D) red blood cell variable at each time point, (E) fibrinogen variable (F) leukocytes variable.

Figure 32 - Clinical laboratory results in equine treated with Nile tilapia fish skin occlusive curative

Source: Cicolo, 2022

2.6 Discussion

NTFS is a promissory tissue to be used as occlusive bandage for equine wounds (13,25). Although the treatment helps in the healing process, it does not replace specific treatment for the primordial disease. In habronema cases ivermectin treatment should be used on the first day. Even treating with ivermectin on day zero, eosinophilia and habronema larvae were found until day 14 on histopathological evaluation. Surgery excision of EGT may be helpful in removing a great part of parasites of the wound spot, also increasing contact surface for NTFS adhesion.

In animal 3 evaluation, a good result was observed NTFS one day after surgical excision of granulation tissue. Effective debridement of chronic wounds is accepted as an essential component of care throughout the wound healing continuum. So debridement can be included before tilapia skin application. Initial wound therapy should be similar to that of a wound to be sutured and should focus on controlling infection (regional limb perfusion, systemic antibiotics) and removal of foreign material and damaged tissue (complete wound excision, lavage), so maybe these procedures can be associated in a forwarding protocol (1,3).

In the first two weeks using NTFS curative, an intensive yellow secretion was observed, at the same time intense neutrophilic infiltrate was observed in microscopy analysis. According to clinical and pathology findings, it seems that chronic stagnant wound changes to active healing and IRA is induced by NTFS. Although the molecular pathways and cytokines involved to start producing this intense secretion and neutrophilic infiltrate remain unclear.

One reason for the formation of exuberant granulation tissue instead of healing is the inability of horses' defense cells to eliminate pathogens. But it is important to declare that tilapia skin has non-pathogenic microbiota, so it is not caused by NTFS contamination, the secretion production is a consequence of IRA induced by NTFS on equine wounds (6,11).

Changes in fibrinogen concentration observed in dialysate in limb wounds did not parallel the changes measured in plasma. Total protein concentrations in body wounds follow the changes in serum total protein, but total protein concentrations in limb wound dialysates were significantly higher than in body wound dialysates, probably due to a nonresolved inflammatory response in the wounds healing with formation of EGT (12).

In the present study, a decrease in plasma fibrinogen media on day 14 was observed, even if no statics changes were noted. According to (12) dialysate in limb wounds did not parallel the changes measured in plasma. Maybe it does occur due to the fact that TNTFS induces IRA and not EGT. An evaluation of limb wound dialyse could bring new information, if it follows normal EGT parameters or IRA parameters, that were observed in equine body wounds. Wound dialyse may also help answer the reason for different results in diverse animals, since there were observed more individual differences in the amount of fibrinogen measured on distal limbs wounds than in truck wound dialyse (12).

Greenhalgh and Singer scales showed a final score lower than the initial score, probably due to the fact that NTFS induces IRA and, maybe to the fact that chronic equine wound follows different healing pathways than humans. According to Bundgaard, 2016 in human concentrations of the APP C-reactive protein were found to decrease in wound exudates when wounds progressed from the non-healing phase to the healing phase, but in horses wound limbs the increase. Probably a new scale needs to be developed for equine chronic wounds evaluation (12,19,20).

The bandagiing protocol can be improved in relation to the one applied in the current study. According to Dr. Edmar's team, human curatives are changed when secretion is observed, as it is considered a sign of contamination. Horses` wounds develop exuberant granulation tissue due to the fact that immunology cell response is not effective for eliminating bacterias. If NTFS curative is changed more frequently in the first two weeks, during IRA response, a faster and better healing process may be more likely. Changing bandages more frequently in the first two weeks may increase IRA response and also might help in controlling the foul smell due to secretion, aiding the prevention of flies and myiasis problems.

Burned humans treated with NTFS had decreased analgesic needs and visual analogue scale. In the present study, this aspect was not evaluated, but a reduction in reactiveness was noticed when handling wounds for bandage change over time which caught the attention of the veterinarians involved. Children treated with tilapia skin showed less pain (7,23,26). Dr. Peyton from UC Davis describes in Healing Burned Animals with NTFS web site that Tilapia skin, like human skin, can transfer collagen, a healing protein. It can reduce pain and also do not cause damage to animals if it is ingested by the patient, unlike ordinary cloth bandages that may cause intestinal obstruction. The animal number 1 ate tilapia skin and, in this study, we didn’t see intestinal obstruction or other problems.

In the same article, was showed that human patients treated with NTFS required fewer days for reepithelialization (9.7 ± 0.6 days versus 10.2 ± 0.9 days; $p = 0.001$) and fewer dressings (1.6 ± 0.7 versus 4.9 ± 0.5; $p < 0.001$). There is no golden standard treatment to compare in equine wounds, but in HOVET USP routine, bandages are normally changed every two days and with tilapia skin curative it was changed after one week. Dr Peyton, from UC Davis, also believes that the low-cost and widespread availability of tilapia skins makes it a game-changer for treating burns, whether animal or human. The observation seems also applicable to horses, although further studies are needed to understand molecular mechanisms such as cytokine patterns, response of macrophages and other cells involved in the healing process when NTFS is used as an occlusive curative (7,23,26).

It is safe to say that essentially all therapies for non healing wounds are used in various combinations because no single treatment is completely therapeutic as a single modality. Animal 7 combined treatment if NTFS and copper sulfate showed a great result, also NTFS probably can be used associated with other therapy protocols such as ozonio therapy, laser therapy etc. Also, in older horses wound delay healing may involve endocrinopathies as pituitary pars intermedia dysfunction or equine metabolic syndrome, so maybe an endocrine evaluation should be also indicated (2,16).

2.7 Conclusion

The present study showed that NTFS had a great wound contraction, showing a good area reduction over a 28 days period. Also NTFS induced AIR which changed EGT formation pathways to the healing process. Improves on bandaging changes protocol could also bring better results. Furthermore, association of NTFS to other therapies could improve equine wounds healing process. Besides that, more studies are necessary to understand molecular pathways of NTFS on the horse wound healing process.

2.7 References

1. Hackett RP. How to Prevent and Treat Exuberant Granulation Tissue. Proc 57th Annu Conv Am Assoc Equine Pract San Antonio Tex USA 18-22 Novemb 2011. 2011;367–73.

2. Maher M, Kuebelbeck L. Nonhealing Wounds of the Equine Limb. Vet Clin North Am Equine Pract. Dezembro de 2018;34(3):539–55.
3. Frykberg RG, Banks J. Challenges in the Treatment of Chronic Wounds. Adv Wound Care. Setembro de 2015;4(9):560–82.
4. Lima Júnior EM, Moraes Filho MO de, Forte AJ, Costa BA, Fechine FV, Alves APNN, et al. Pediatric Burn Treatment Using Tilapia Skin as a Xenograft for Superficial Partial-Thickness Wounds: A Pilot Study. J Burn Care Res. 26 de Agosto de 2019;irz149.
5. Lima Júnior EM, De Moraes Filho MO, Costa BA, Rohleder AVP, Sales Rocha MB, Fechine FV, et al. Innovative Burn Treatment Using Tilapia Skin as a Xenograft: A Phase II Randomized Controlled Trial. J Burn Care Res. 2 de Maio de 2020;41(3):585–92.
6. Lima Junior EM, Bandeira T de JPG, Miranda MJB de, Ferreira GE, Parente EA, Piccolo NS, et al. Characterization of the microbiota of the skin and oral cavity of Oreochromis niloticusCaracterização da microbiota da pele e cavidade oral de Oreochromis niloticusdoi:10.12662/2317-3076jhbs.v4i3.767.p193-197.2016. J Health Biol Sci. 28 de Setembro de 2016;4(3):193.
7. Lima Júnior, Edmar Maciel, e Moraes Filho, Manoel Odorico, Costa, Bruno Almeida, Fechine, Francisco Vagnaldo, Vale, Mariana Lima, Diógenes, Ana Kely de Loyola, et al. Nile Tilapia Fish Skin–Based Wound Dressing Improves Pain and Treatment-Related Costs of Superficial Partial-Thickness Burns: A Phase III Randomized Controlled Trial. Plast Reconstr Surg. Abril de 2021;147(5):1189–98.
8. Barrientos S, Stojadinovic O, Golinko MS, Brem H, Tomic-Canic M. PERSPECTIVE ARTICLE: Growth factors and cytokines in wound healing: Growth factors and cytokines in wound healing. Wound Repair Regen. Setembro de 2008;16(5):585–601.
9. Caron, J.P. Management of superficial wound. Em: Equine Surgery. 1.ª ed. 1999. p. 129–40.
10. Hendrickson D, Virgin J. Factors that affect equine wound repair. Vet Clin North Am Equine Pract. Abril de 2005;21(1):33–44.
11. Wilmink JM, van Weeren PR. Differences in wound healing between horses and ponies: Application of research results to the clinical approach of equine wounds. Clin Tech Equine Pract. Junho de 2004;3(2):123–33.
12. Bundgaard L, Bendixen E, Sørensen MAa, Harman VM, Beynon RJ, Petersen LJ, et al. A selected reaction monitoring-based analysis of acute phase proteins in interstitial fluids from experimental equine wounds healing by secondary intention: Selected reaction monitoring-based analysis of acute phase proteins in wound interstitial fluids. Wound Repair Regen. Maio de 2016;24(3):525–32.
13. Costa BO, Lima Júnior EM, Fechine FV, Nunes Alves APN, Oliveira de Melo MM, Correia Ribeiro WL, et al. Treatment of a Traumatic Equine Wound Using Nile Tilapia (Oreochromis niloticus) Skin as a Xenograft. Acta Sci Vet [Internet]. 3 de Junho de 2020 [citado 6 de Dezembro de 2021];48. Disponível em: https://seer.ufrgs.br/ActaScientiaeVeterinariae/article/view/99678
14. Jacobs KA, Leach DH, Fretz PB, Townsend HGG. Comparative Aspects of the Healing of Excisional Wounds on the Leg and Body of Horses. Vet Surg. 28 de Junho de 2008;13(2):83–90.
15. Knottenbelt DC. Equine wound management: are there significant differences in healing at different sites on the body? Vet Dermatol. Dezembro de 1997;8(4):273–90.
16. Frank N. Advancing research and providing recommendations for the diagnosis and management of equine endocrine disorders. [Internet]. Equine Endocrinology Group. 2017. Disponível em: https://sites.tufts.edu/equineendogroup/
17. Martin-Gallausiaux C, Larraufie P, Jarry A, Béguet-Crespel F, Marinelli L, Ledue F, et al. Butyrate Produced by Commensal Bacteria Down-Regulates Indolamine

2,3-Dioxygenase 1 (IDO-1) Expression via a Dual Mechanism in Human Intestinal Epithelial Cells. Front Immunol [Internet]. 11 de Dezembro de 2018;9. Disponível em: https://www.ncbi.nlm.nih.gov/pmc/articles/PMC6297836/

18. Darwin E, Tomic-Canic M. Healing Chronic Wounds: Current Challenges and Potential Solutions. Curr Dermatol Rep. Dezembro de 2018;7(4):296–302.

19. Singer AJ, Thode HC, McClain SA. Development of a Histomorphologic Scale to Quantify Cutaneous Scars after Burns. Acad Emerg Med. Outubro de 2000;7(10):1083–8.

20. Greenhalgh DG, Sprugel KH, Murray MJ, Rosst R. PDGF and FGF Stimulate Wound Healing in the Genetically Diabetic Mouse. 1990;136(6):12.

21. Alves APNN, Lima Júnior EM, Piccolo NS, de Miranda MJB, Lima Verde MEQ, Ferreira Júnior AEC, et al. Study of tensiometric properties, microbiological and collagen content in nile tilapia skin submitted to different sterilization methods. Cell Tissue Bank. Setembro de 2018;19(3):373–82.

22. Lima-Junior EM, Picollo NS. The use of tilapia skin (Oreochromis niloticus), as an occlusive biological dressing, in the treatment of burn wounds. :8.

23. tdus. Healing Burned Animals with Fish Skins [Internet]. UC Davis. 2018 [citado 28 de Janeiro de 2022]. Disponível em: https://www.ucdavis.edu/health/news/healing-animals-with-fish-skins

24. Bunn A, Korpela M. An Introduction to dplR. :16.

25. Silva SC da, Ríspoli VFP, Graner C, Sá LRM de, Belli CB, De Zoppa ALDV. Using tilapia skin (Oreochromis niloticus) as an occlusive biological curative in equine wounds: Braz J Vet Res Anim Sci. 16 de Janeiro de 2020;56(4):e154079.

26. Costa BA, Lima Júnior EM, de Moraes Filho MO, Fechine FV, de Moraes MEA, Silva Júnior FR, et al. Use of Tilapia Skin as a Xenograft for Pediatric Burn Treatment: A Case Report. J Burn Care Res. 14 de Agosto de 2019;40(5):714–7.

3 GENERAL CONCLUSION

Equine wounds are an important topic to be discussed in the veterinary routine. Many protocols are described and applied for treating chronic wounds in horses, although there is not any goldstandt. Due to the complicated healing process in this species, a long period of hospitalization and expensive costs for owners are still necessary.

Different techniques are associated in hospital routine to have a successful result. Biologic membranes such as pericard and amniotic horse membrane have great results, however, with difficult access due to contamination. Also studies are showing great results using occlusive curatives.

Tilapia skin is a great option, due to being easy to access and a non-expensive product. Recently studies showed great results using NTFS for different kinds of wounds in humans.

The results of this study highlighted that it is possible to use NTFS as an occlusive bandage in horses, with great results in the healing process. Adjustments, however, are still necessary in application protocol for better results. It was observed that NTFS induces an IRA on equine wounds, but also more studies need to be done to understand molecular pathways of NTFS process in equine wound healing.

NTFS is not commercially available yet, tilapia skin found on farm markets can be used following protocol applied in the two pilot cases. NTFS is of easy access in Brazil, often considered waste and offered for free.