Healing efficacy of a novel wound therapy with enrofloxacin-HCl·2H_2O/sodium alginate in unresponsive wounds in dogs

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Abstract

Wound-healing of lesions unresponsive to antibiotic treatment in dogs prompted a clinical trial aimed to assess a new hydrogel based on sodium alginate and the enrofloxacin crystal-solvate (HCl·2H_2O [enro-C]). Fifty-six cases of full-thickness infected cavity wounds, unresponsive to at least one complete antibiotic treatment scheme, were included in this trial over a year. Patients were classified into three severity categories based on their lesions' chronicity and clinical characteristics. The hydrogel was applied twice to four times daily for the necessary time until resolution (endpoint). Follow-up was done every other day for up to 4 weeks when required and weekly for two more months after the endpoint was reached. The hydrogel was internalized in the cavity wounds, delivering the necessary volume which was becoming progressively smaller until healing as perceived by clinicians and owners. A control group was formed based on historical data from the participants' case filings. All dogs in the trial healed. The mean time needed for full recovery was 6.75 ± 1.36 days in dogs graded severity-1, 13.76 ± 4.2 days for severity-2, and 24.47 ± 6.7 days for severity-3. Tissue concentrations of enro-C/gram of wounded tissue must be determined to improve and rationalize the use of the tested hydrogel, and systemic and topic drug interactions must be determined. The outstanding biocompatibility, gel-forming ability, and lack of apparent toxicity of the hydrogel make it an advantageous option for wound treatment.

Canine, skin injury, alginate, biopolymer, cicatrization

Skin lesions and injuries that reach deeper tissues are often attended to at the veterinarian's office. Based on the bacterial load, they can be classified as clean, clean-contaminated, contaminated, and dirty. The bacterial load then provides the key to the therapeutic approach. In veterinary medicine, unless a lesion comes from surgical intervention, most wounds are contaminated, considering that an infected wound has a bacterial load > 1×10^5 colony-forming units/gram of tissue (Dow et al. 1999; Landis 2008). Often, cavity wounds are classified as contaminated; such is the case of deep bites, injuries from a sharp object, skin avulsion due to trauma, and some cases caused by oncological surgery in which a large amount of necrotic tissue impedes healing (Del Villar et al. 2012; Negut et al. 2018). In clinical work with companion animals, many wounds can be classified as chronic and are challenging due to their slow progression. Chronic cavity wounds are common in daily veterinary clinical work. Systemic and/or topical treatment with antibacterial drugs and wound-repairing agents is a rational option. In these types of wounds, the rate of blood supply, the degree of cavitation of the lesion, and the amount of necrotic tissue are crucial factors that influence the course of healing (Kujath and Michelsen 2008; O'Dwyer 2010). However, other factors may interfere with the efficacy of treating such lesions, i.e., full compliance with treatment, bacterial resistance, and underlying health conditions such as diabetes, malnutrition, and obesity.

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An approach for managing such chronic wounds is using topical dressings. Such potential materials are the biopolymers, which can also include or carry a potent antibacterial drug and allow its sustained release. The chosen biopolymer should maintain a moist environment, allow oxygen passage and angiogenesis, sustain epidermal migration of cells, and stimulate the synthesis and organized deposit of connective tissue without causing allergic reactions (William and Eaglstein 2008; O'Dwyer 2010). At the Department of Physiology and Pharmacology at the National Autonomous University of Mexico (UNAM), a solvate derivative of enrofloxacin (enrofloxacin $HCl{\cdot}2H_2O$ = enro-C) has been patented (Patent 472715: Instituto Mexicano de la Protección Industrial, Mexico City, December 11, 2013). This recrystallized form of enrofloxacin is uniquely compatible with sodium alginate as it can be incorporated to obtain a hydrogel preparation ($HCl{\cdot}2H_2O$-sodium alginate hydrogel [Al/enro-C]). Preliminary results with a similar hydrogel have been encouraging in experimental rats (Del Villar et al. 2012), and deep pyoderma in dogs (Gutierrez et al. 2020). Considering the above, the working hypothesis of this open clinical trial was to assess if a wide array of contaminated wounds in dogs, refractory to either systemic or local antibiotic therapy at least on one occasion, will benefit from the topical administration of a hydrogel based on enro-C in alginate.

Materials and Methods

Ethics statement

The study design and animal handling complied with the Mexican regulations for using experimental animals as established by the National Autonomous University of Mexico (UNAM) and the Mexican standards established in NOM-062-ZOO-1999 and CICUA-FMVZ-UNAM. All the investigations and treatments were performed as necessary for each patient's best interest. The owner's written permission was obtained to use images and clinical records for publications and educational purposes.

The clinical trial was conducted in collaboration with ten clinics or hospitals for small animals in Mexico City. The work was undertaken from January 2022 to February 2023. The Support Program for Research and Technological Innovation Projects (PAPIIT, Number ID: IT200222, National Autonomous University of Mexico (UNAM) approved and supported this research. The Internal Committee of Postgraduate Studies of the School of Veterinary Medicine of the Universidad Nacional Autónoma de Mexico approved the ethics procedures followed in this study (CICUAL-DC-2018/2-1).

Animals and procedures

In total, 56 dogs of both sexes and different breeds were included and closely followed up through observational exams by trained clinicians unaware of the hydrogel's contents. They all suffered from chronic, bacterially infected wounds characterized by pruritic and swollen lesions, a certain amount of fur loss, open wounds often with necrotic edges, pain, purulent discharge, and a foul odour. The only common link among these lesions was that they were unsuccessfully treated with antibiotic therapy administered for systemic effect on at least one occasion. Table 1 summarizes the main features of the Al/enro-C hydrogel treated dogs and the real-world historical data from dogs with similar medical histories retrieved from participant clinicians' files was included to set a control group with the standard of care (Liu and Panagiotakos 2022).

Before commencing this clinical trial, a 5-day washout period of previous medications was imposed in all cases. No systemic treatment was attempted, and no other drug was allowed during this trial. Before the initial treatment, skin swabs were obtained in all cases for microbiological analysis at the Microbiology Laboratory at UNAM. However, the characterization of sensitivity/resistant patterns was not attempted. No changes in diet or housing were imposed or suggested. Weekly baths with a neutral-pH shampoo (Mennen zero%®, Colgate-Palmolive Co., Mexico City, Mexico) free from alcohol, dyes, silicones, perfume, selenium, fatty acids, and with a pH = 6 were allowed. Treatment was carried out only with enro-C hydrogel in an alginate base. In all cases, the hydrogel was applied to the wounds at least two and preferably four times daily. The hydrogel dose or schedule to smear it was not constant as owners applied it at home. Owners were advised to use gloves to smear or apply the hydrogel. In most cases, the hydrogel dries out on the wounds in less than 15–20 min, and patients require no bandages.

Furthermore, it induced a soothing effect, and collars were regarded as unnecessary. On four occasions, the gel was inserted by a veterinary surgeon into a forming abscess or a cavity wound, ensuring that an opening allowed debridement.

This study can be described as an open-label longitudinal clinical trial. The dogs included in this study had no direct comparison with control dogs simultaneously. Instead, historical clinical data from 41 dogs with similar medical histories were retrieved to set a case-control group with the standard of care (Liu and Panagiotakos 2022). The reasons for this decision were the wide variety of clinical scenarios, i.e., the nature of wounds, general care

conditions of each patient at home, breeds, and feeding habits that were unique to each individual. Also, assigning some cases randomly to a conventionally treated group was considered unethical and unsustainable, given that all dogs had a previous story of unsuccessful treatment and owners were unwilling to repeat a similar conventional treatment scheme (Miller and Brody 2002; Paulus et al. 2014; Kramer and Font 2017). Experimental and control dogs were divided according to their clinical signs into moderate, severe, and very severe cases, based on the criteria presented in Table 2 (Kujath and Michelsen 2008; William and Eaglstein 2008; O'Dwyer 2010). A complete clinical history was obtained and is available for each case, including renal, hepatic, and haematological profiles. These tests were repeated at the end of the treatment. Exclusion criteria included kidney or liver insufficiencies to minimize bias, neoplastic or autoimmune pathologies such as callus pyoderma, parasitic dermatoses, fungal infections, leishmaniasis, hyperadrenocorticism, growth hormone deficiency, diabetes mellitus, allergic pruritus, cutaneous neoplasia, juvenile cellulitis, calciphylaxis due to end-stage renal disease and hyperparathyroidism, immunomodulatory-responsive lymphocytic-plasmacytic pododermatitis, pemphigus foliaceous, pyoderma gangrenosum, and other unidentified pathologies. Hormonal level studies were performed only when some associated endocrine pathology was suspected and in order to exclude the patient from this trial if it was positive. Daily follow-ups were ensured by the attending clinician and by one of the authors every other day until resolution, which was regarded as treatment success or failure. The assessment of improvement or lack thereof was carried out every other day, and it was based on observations gathered from each of the trained veterinary surgeons participating in this study together with one of the authors.

After the dogs were declared clinically cured, as perceived by the endpoint criterion detailed below, a two-month visual/clinical follow-up was established to detect any adverse effects due to the use of hydrogel or whether an infection was slowly progressing. No bacteriological follow-up was attempted during this period. The endpoint to declare a patient clinically healed was established based on the lack of clinical signs, i.e., pain or discomfort, foul odour, secretions, pruritus, and by visual and tactile evaluation confirming that the affected body area was

Table 1. Summary of cases of infected wounds or lesions in 56 dogs, unresponsive to at least one antibacterial drug(s) treatment* and treated with the experimental alginate/enro-C hydrogel.

Severity grade	Number of cases	Age (Mean ± SD)	Main antibiotic previously used[#]	Main causative/associated pathogen
Treated with Alginate/enro-C hydrogel				
1	12	5.03 ± 3.6	8 AmC; 2 Enr; 1 Cefv; 1 Cef	2 *Pasteurella multocida,* 1 *Prevotella* spp, 2 *Streptococcus canis*, coagulase-neg; 4 *Staph.* spp, 2 *Staphylococcus intermedius*, 1 *Fusobacterium* spp.
2	25	6.4 ± 2.5	15 AmC; 4 Enr; 3 Marb; 3 Cefv	3 *Pasteurella multocida,* 3 *Prevotella* spp, 4 *Streptococcus canis*, coagulase-neg; 8 *Staph.* spp, 5 *Staphylococcus intermedius*, 2 *Fusobacterium* spp
3	19	7.2 ± 3.8	11 AmC; 4 Enr; 4 Cefv	5 *Streptococcus canis*, coagulase-negative; 8 *Staph.* spp, 6 *Staphylococcus intermedius*
Real-world historical data•				
1	9	5.33 ± 2	3 Enr; 10 Cefv; 1 Cef	3 *Pasteurella multocida,* 3 *Streptococcus canis*, coagulase-negative; 4 *Staphylococcus intermedius*; 2 *Fusobacterium* spp.
2	16	6 ± 1.96	8 AmC; 4 Enr; 4 Marb	1 *Pasteurella multocida,* 2 *Prevotella* spp, 3 *Streptococcus canis*, coagulase-negative; 10 *Staphylococcus intermedius*
3	16	6.75 ± 1.6	6 AmC; 4 Enr; 6 Cefv	2 *Pasteurella multocida,* 3 *Prevotella* spp, 3 *Staphylococcus* species, 8 *Staphylococcus intermedius*

*All lesions had been previously treated with one or two of the following antiseptics: chlorhexidine, povidone-iodine, chloroxylenol, isopropyl alcohol, hexachlorophene, benzalkonium chloride, and hydrogen peroxide.

[#]AmC = amoxicillin plus potassium clavulanate; Enr = enrofloxacin; Cefv = cefovecin; Cef = cefalexin; Marb = marbofloxacin.

• Real-world historical data from dogs with similar medical histories retrieved from participant clinicians' files were used to create the control group. Clinical histories were retrieved from participant clinicians' files from 2020 to date.

repaired and a visually healthy tissue was evident. Most owners were reluctant to cooperate to carry out blood tests at the end of the trial. Hence, these data are missing in this study.

Al/enro-C was manufactured at the Laboratory of Pharmacology of the UNAM under good manufacturing practices according to the following composition: 0.8% enro-C in 2% sodium alginate and 0.5% propylene glycol. The gel was manufactured every month and poured into 80 ml plastic bottles, which were kept refrigerated at all times (4 °C). Enro-C batches were prepared as indicated in the corresponding patent.

For statistical analysis, clinical signs before treatment and after healing were compared using Wilcoxon matched-pair rank test with Z approximation. Progression was recorded every three days, and Kaplan Meier Log-rank analysis was done to compare the cumulative progression of all five groups (the control group with severity grade 3 had none of the dogs cured). Kruskall-Wallis test of days to the endpoint was carried out for the cured dogs. The IBM SPSS package was used, and no deviations from the set protocol occurred.

The general characteristics of the dog population included in this study, the number of animals associated with each degree of open-infected wound severity, and the possible associated pathogens are presented in Table 1. The severity of the cases was adjusted to the criteria laid out in Table 2. As the classification of wounds varies among authors, in this trial, in addition to the direct aspect of the injury, the following were considered to add-up subjectively to classification of the severity level, i.e., the cause of injury (trauma, surgery, chronic diseases), the duration (acute or chronic), the depth of injury (superficial, partial thickness or deep dermal injury involving epidermis, dermis, and underlying tissues), and the time elapsed from when the injury originated until the primary medical intervention (Kordestani 2019). Also, given the home-care working model of this trial and the heterogeneity of the wounds and settings involved, a perceptual assessment of the amount of Al/enro-C hydrogel utilized was adopted, added to observing the residual volume of hydrogel left in the container. Given that these measures are subjective, they were not quantified.

Results

All dogs included in this trial recovered entirely from their infected unresponsive-to-treatment wounds. The mean time needed for full recovery was 6.75 ± 1.36 days in dogs graded 1 for severity, 13.76 ± 4.2 days for severity-2, and 24.47 ± 6.7 days for severity-3. Figure 1 shows the cumulative progression of cases healed every three days. As an example of the noticeable tissue repairing capability of the tested hydrogel, Fig. 2 (Plate III) presents the treatment of a severely affected nose of a Collie dog with marked loss of tissue and having failed to treatment with either amoxicillin/potassium clavulanate or enrofloxacin (severity grade-3; Fig. 2A), and the tissue repairing process that occurred after 33 days of treatment (Fig. 2B). In this particular case, treatment was extended for 15 additional days after the condition of the dog's nose was inspected, and without other reason but to avoid potential relapse. It is important to note that in Fig. 2A, although the patient's face has less ambient lighting, it is noticeably swollen. Figure 3 (Plate III) shows a dog with a recurrent phlemonic ventral abscess caused by partial mastectomy. The Al/enro-C hydrogel was internalized into the wound until saturated (Fig. 3A), using a needleless syringe. Fig. 3B shows the same patient 30 days later. In Fig. 3C, a close-up shows one of the wound openings now closed. It is essential to emphasize that the hydrogel was not force-inserted into the wound during treatments. Thus, administration toward the end of the treatment was achieved by smearing the hydrogel in the closing wound.

In all cases, the hydrogel's smearing or internalization caused no stress. The gel causes enough well-being in all patients to allow for the perception of a contrasting experience with the typical application of skin antiseptics that induce unpleasant/pungent sensations.

Figure 4 shows the median ± 1 SD of days needed to heal bacterially infected refractory wounds both in the hydrogel treated group and in the control one. Since the time variable does not have a normal distribution and in congruency with the chosen test, medians were utilized instead of means. Significant differences between groups after Kruskall-Wallis test are denoted by different letters. Cumulative progression of chronic wounds as recorded every 3 days both in the referred groups is depicted in Fig. 5 (Plate IV) based on Kaplan Meier LogRank ($P < 0.0001$).

The data supporting this study's findings are available from the corresponding author upon request.

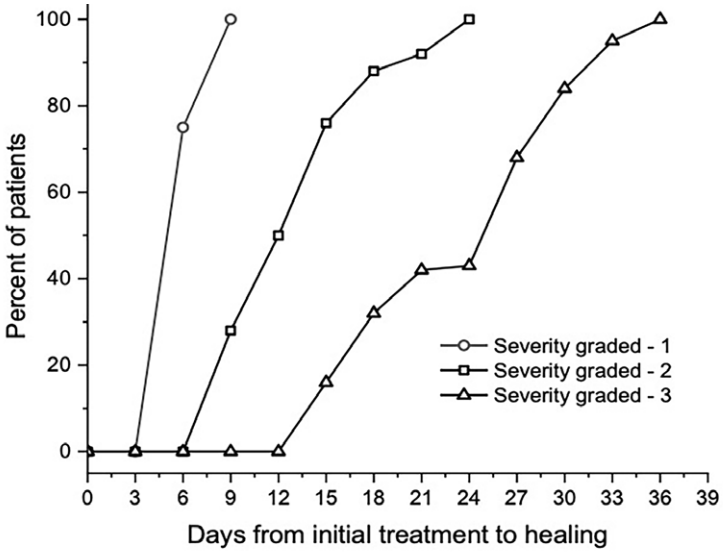

Fig.1. Cumulative progression of chronic wounds, bacterially infected refractory at least to one treatment with antibiotics and finally healed with the hydrogen manufactured with alginate and euro-C. Progression was recorded every three days.

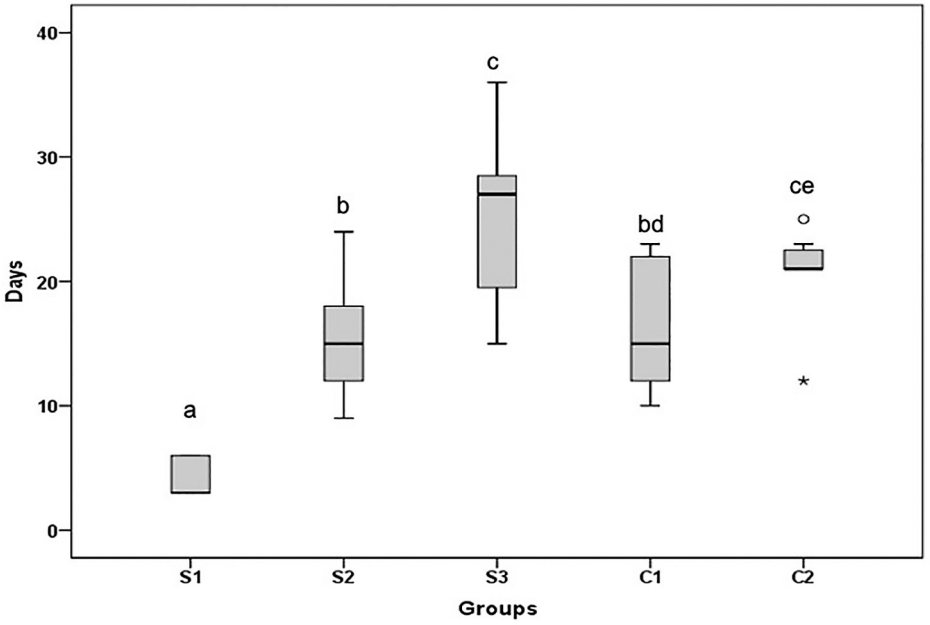

Fig. 4. Days needed to heal bacterially infected refractory wounds.

S1: severity-1 grade group of dogs; S2: severity-2 grade group of dogs; S3: severity-3 grade group of dogs; C1, C2: control groups

Discussion

In small animal practice, most wounds heal without complications, as tissue restoration is the body's natural tendency. However, wounds are occasionally neglected. When a wound is infected and the owner fails to promptly detect it, bacteria gain access into deep tissues. If the blood supply to the area is deficient, a contaminated, cavity wound is likely to develop. Treatment with antibacterial drugs locally or systemically or both, having removed all contaminated or necrotic tissue, usually resolves many cases. However, unresponsive, full-thickness, open wounds may develop, causing significant tissue damage and turning wound healing into a long-lasting and challenging process (William and Eaglstein 2008; O'Dwver 2010; Wilkins and Unverdorben 2013). In some cases, surgical intervention is regarded as necessary, and long-lasting treatments with various local and systemic antibiotics are imposed. Also, wound dressings are generally applied. These dressings can be classified as passive, interactive, and bioactive products based on their degree of tissue vs. material interaction (Ishihara et al. 2001; Sheskin 2011).

Skin continuity of open chronic wounds usually takes 30–45 days and sometimes more extended periods depending on many variables such as nutrition status, housing conditions, individual traits, etc. In most cases, antibiotics are administered in open wounds that show great loss of tissue. The newly formed granulation tissue must be preserved and thorough assessment of further intervention must be made during the following weeks to ensure a satisfactory development. Of course, surgical debridement must be performed in the face of necrotic tissue. The decision as to whether or not to surgically remove the newly formed tissue involves many aspects, such as the subjective evaluation of the clinician, which should include the animal's welfare and the owner's consent.

Bacterial load is present in open wounds; biofilm has been found in 60% of unresponsive open wounds, which is paramount to be addressed. Bacterial contamination, colonization, and establishment of the infection are dynamic processes, and the exact role of the Al/enro-C hydrogel used in this trial remains to be clarified. However, this must be done outside a clinical trial (Daeschlein 2013) to assess whether or not an intervention could destroy the necessary initial repairing tissue needed for wound healing. We must emphasize that under real-life clinical conditions in which this trial was based, it was impractical for owners to attend the hospital routinely with their pets to have indices such as wound surface area, wound contraction, or degree of inflammation determined. Many of these dogs had undergone long processes and the owners of most of these patients were more inclined to refuse any further treatment. Hence, they were granted treatment and follow-up at no cost, and a signed consent was required stating that the option for euthanasia was at the owners' discretion.

In this trial, the healing capabilities of the new hydrogel, a bioactive product (Al/enro-C hydrogel), were utilized without systemic antibacterial drug support. It is worth noting that a control group in this type of trial can be regarded as unethical, considering that owners of each wounded dog were seeking an alternative treatment given that previous treatments based on conventional antibiotics and dressings had failed (Paulus et al. 2014; Kramer and Font 2017). In any case, within the setting of this trial, their previous medical treatments can be considered as external controls, i.e., patients treated earlier (EMEA 2001), allowing a comparison of the outcomes caused by the test treatment with those of the failed previous treatments.

Wound dressings based on alginate material are well-known in the literature (Piacquadio and Nelson 1992; Sedlarik and Nusser 1992; Seymour 1997), and various commercial preparations are available for wound management. However, the enro-C-sodium alginate hydrogel utilized in this trial is unique as the chemical compatibility of sodium alginate was only achieved with this new crystal solvate of enrofloxacin and

not with standard enrofloxacin or its HCl derivative or any other fluoroquinolone tested. It is postulated that Al/enro-C hydrogel achieves a sustained release of enro-C that lasts three to six hours, depending on when it dries. Similar behaviour has been described for various drugs in alginate (Momoh et al. 2015), including ciprofloxacin (Han et al. 2014) and Ag-sulphadiazine (Boateng et al. 2015). These results are not shown in this clinical trial. Similarly, this trial's clinical nature was incompatible with the chemical determination of enro-C in samples of the affected tissue to establish a concentration ratio of the antibacterial per gram of affected tissue. However, congruency of MIC vs enro-C concentrations is foreseen, given the obtained results. One obvious advantage of the Al/enro-C hydrogel is that it acts as a natural haemostat. Also, the gel-forming property of alginate helps remove the dressing without much trauma; in most cases, this procedure was unnecessary. It became apparent to clinicians that the hydrogel tested reduced the pain the patients experienced, a feature already established for humans with other alginate-based dressings (Odonoqhue et al. 1997; Sahoo and Biswal 2021). Nevertheless, the overlap of wound healing phases in patients with unresponsive wounds, such as synthesis of growth factors, presence of cytokines and chemokines, degree of inflammation, re-epithelialization, wound contraction, and maturation phases, avoided further measurements of the progress of the healing (Balsa and Culp 2015; Pavletic 2018; Lux 2022).

The experimental nature of this clinical trial was based on previous experiences in treating deep-pyoderma unresponsive cases in dogs (Gutierrez et al. 2020). Although detailed microbiological studies of susceptibility and bacterial resistance were not carried out due to insufficient funds, it is safe to assume that many of these cases treated with Al/enro-C hydrogel could have fallen under the assumption of enrofloxacin resistance (Yoo et al. 2010). As already stated, these studies are currently being carried out to establish the consistency between tissue concentrations of enro-C and MICs and resistance patterns observed at the laboratory level.

The study has shown that sodium alginate, a polysaccharide of natural origin, was chosen to manufacture the $HCl{\cdot}2H_2O$-alginate hydrogel based on its proven outstanding biocompatibility properties, gel-forming ability, non-toxicity, biodegradability and easy processing. Additionally, the results suggest that some form of tissue engineering occurs (Plate III, Fig. 2) (Sahoo and Biswal 2021) as it has been postulated that alginates possess a potential for protein delivery in wound healing (Han et al. 2014). This latter assumption should be further sustained and characterized. Finally, drug interactions must be considered if supportive systemic antibiotic treatment is deemed necessary. For example, antagonism between bacteriostatic and bactericidal drugs has been demonstrated, and care should be exercised to avoid using this $HCl{\cdot}2H_2O$-alginate hydrogel with systemic tetracyclines (Han et al. 2014).

Conflict of interest

The authors declare that they have no potential source of conflict of interest or financial relationship of any other type, which could be perceived as an influence on the authors' objectivity in the publication of this paper.

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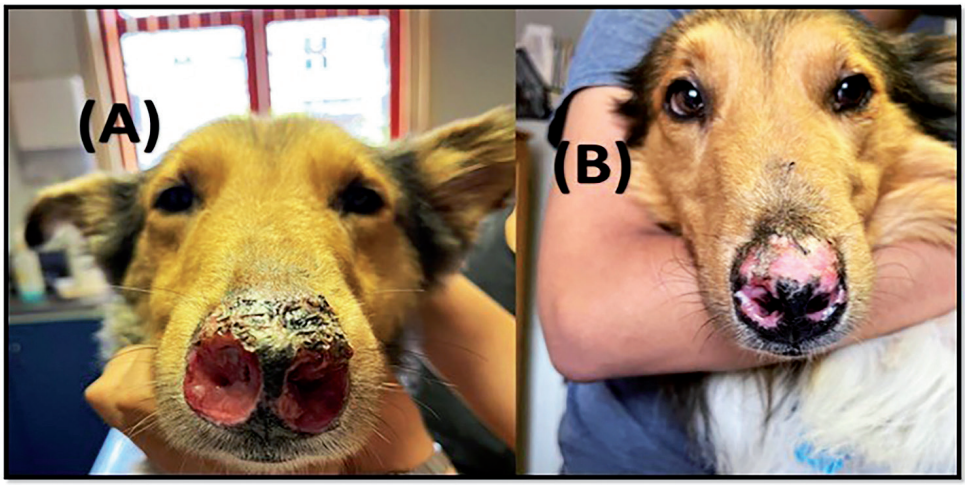

Fig. 2. Aspects of a dog's nose fasciitis with severe tissue loss (severity grade-3) (A) and the recovery observed after 30 days of treatment with enrofloxacin $HCl \cdot 2H_2O$-alginate hydrogel (B)

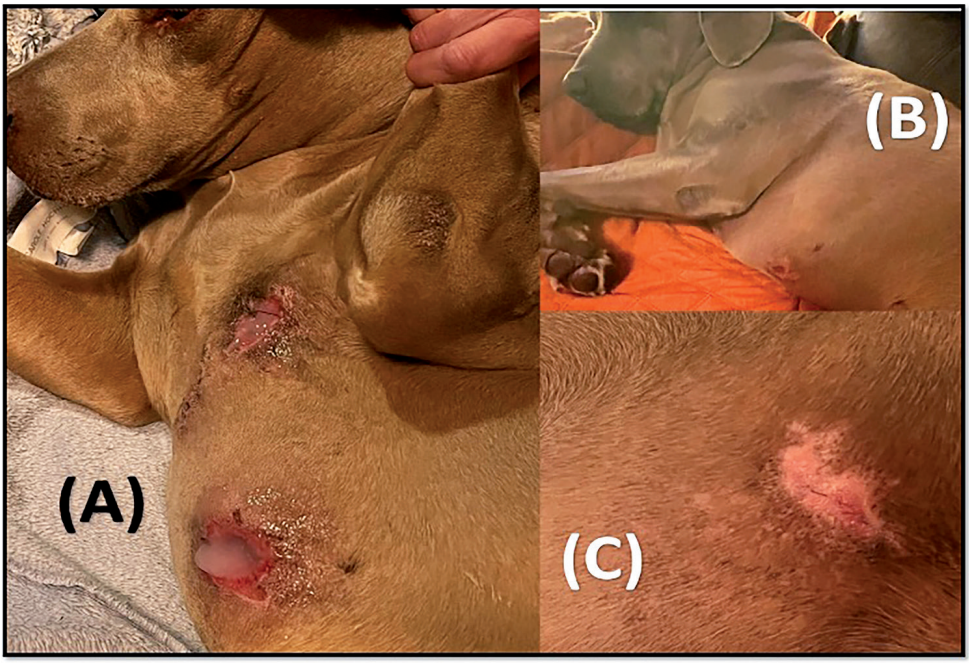

Fig. 3. Dog with a recurrent phlemonic ventral abscess caused by partial mastectomy: (A) internalizing the Al/enro-C hydrogel into the wound; (B) the patient 30 days later; (C) one of the wound openings now closed.

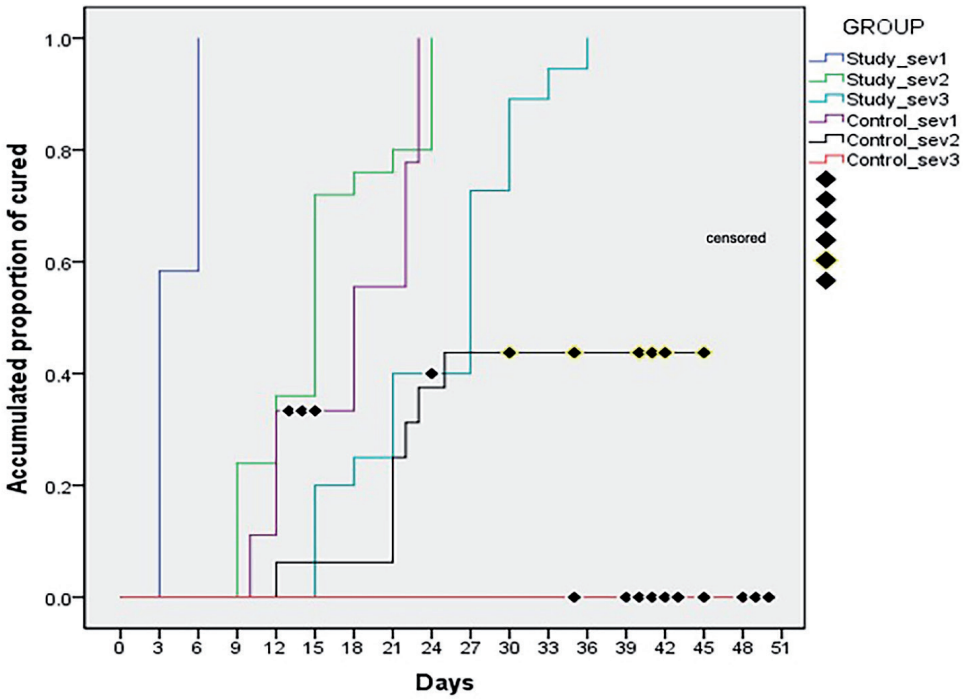

Fig 5. Kaplan Meier LogRank ($P < 0.0001$) cumulative progression of chronic wounds, bacterially infected refractory at least to one treatment with antibiotics with progression recorded every three days.

Study_sev1, Study_sev2, Study_sev3: study groups according to the severity grade that finally headed with the alginate/enro-C hydrogel. Control_sev1, Control_sev2, Control_sev3: progression of control groups according to the severity grade with second intention conventional treatment.