Development of a porcine hard-to-heal wound model: evaluation of a bromelain-based enzymatic debriding agent

Aims: We describe the development of a novel porcine eschar model and compare the debridement efficacy of various concentrations of a novel bromelain-based enzymatic agent with collagenase.

Methods: Full thickness excisional wounds were created on pigs and injected intradermally with various doses of doxorubicin. Wounds were monitored for a period of 46 days for the development of eschar and wound closure. After determining the optimal concentration and dose of doxorubicin resulting in non-healing eschars, these conditions were used to create additional wounds on another set of animals. The resulting eschars were treated with various concentrations of a novel bromelain-based enzymatic agent (EscharEx-02) or collagenase. The primary endpoint was greater than 95% removal of the central eschar.

Results: Consistent eschars composed of two distinct areas (a central area of exudate and slough representing the hard-to-heal wound bed, and a peripheral area of full-thickness mummified necrosis) were seen after injection of doxorubicin (0.5 ml/cm^2 of stock solution 0.75mg/ml) at one and six days after wound creation. Complete removal of the central eschar was achieved in all wounds after five and eight treatments with 5% and 2% EscharEx-02 respectively. Complete removal of the central eschar with collagenase was achieved in 0% and 82% of the wounds after 10 and 16 treatments respectively.

Conclusions: We describe a porcine model for creating eschars similar to hard-to-heal wounds in humans. A novel bromelain-based enzymatic debridement agent was more effective than a commercially available collagenase in removing eschars in this wound model.

Declaration of interest: YS is a consultant for MediWound. The authors have no other conflicts of interest to declare.

bromelain • collagenase • debridement • eschar • hard-to-heal • necrosis • porcine • research • wound • wounds • wound healing

With over six million hard-to-heal wounds reported annually in the US alone, they are a major healthcare burden.[1] Despite the high number of such wounds, there have been few advances in therapies. Hard-to-heal wounds or ulcers are characterised by the presence of an eschar of necrotic tissue and slough, comprising materials secreted from the wound, that delays healing and increases the risk of infection.[2] A cornerstone of therapy is debridement of all eschars. Apart from sharp mechanical debridement, autolytic and enzymatic debridement have been used.[3] However, these processes are slow and only minimally effective.

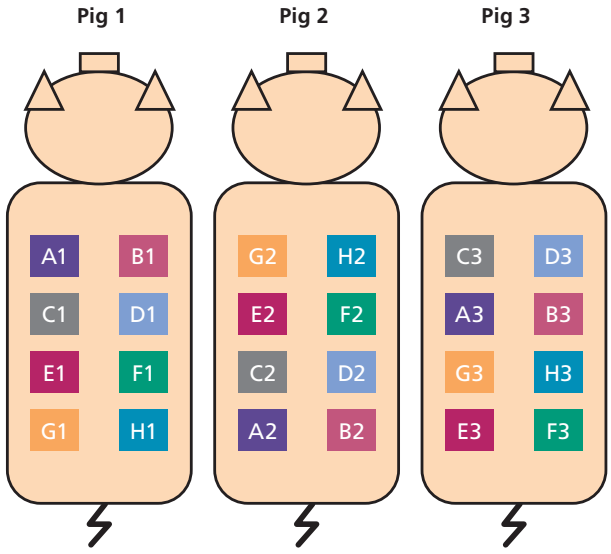
A major barrier to the development of novel therapies for hard-to-heal wounds has been the lack of experimental models, especially in large animals such as the pig.[4] The pig is the preferred animal model for cutaneous research and drug development due to its similarity to the human skin structure.[5] However, in spite of the structural and physiological similarities, wounds in pigs heal faster than in humans, even in the presence of necrotic tissue, limiting their use in hard-to-heal wound research. Doxorubicin is a chemotherapeutic agent used for the treatment of a variety of solid and haematopoietic tumours. One of its well-known adverse effects is skin necrosis after soft tissue extravasation.[6] While the exact mechanisms of skin necrosis are unknown, the molecular and cellular effects are numerous.[7, 8] Some authors have hypothesised that doxorubicin interferes with nuclear function and cell replication by binding to DNA.[9] Others have demonstrated depletion of the reductive capacity of the skin, which may represent another harmful effect of doxorubicin on the skin.[10] The delay in wound healing may also be the result of delayed wound contraction due to doxorubicin's interference with intracellular

Yaron Shoham,[1] MD; **Itai Sabbag**,[2] DMV; **Adam J Singer**,[3] MD*

***Correspondence author email:** adam.singer@stonybrook.edu

1 Department of Plastic and Reconstructive Surgery, Soroka Medical Center, Ben-Gurion University, Beer-Sheba, Israel. **2** Lahav Research Institute, Kibbutz Lahav, Israel. **3** Department of Emergency Medicine, Renaissance School of Medicine at Stony Brook University, Stony Brook, NY, US.

Fig 1. Description of treatment assignment of model development



A (1,2,3): Control, untreated wound
B (1,2,3): Control, untreated wound
C (1,2,3): 0.25mg/ml doxorubicin 2.4ml
D (1,2,3): 0.25mg/ml doxorubicin 4.8ml
E (1,2,3): 0.5mg/ml doxorubicin 2.4ml
F (1,2,3): 0.5mg/ml doxorubicin 4.8ml
G (1,2,3): 0.75mg/ml doxorubicin 2.4ml
H (1,2,3): 0.75mg/ml doxorubicin 4.8ml

contractile myofilaments in skin fibroblasts as seen in cardiac myocytes.[11]

Rudolph et al. developed an experimental model of skin necrosis in rats that was produced by intradermal injection of doxorubicin.[12] They found that doxorubicin consistently produced skin necrosis that was dose-dependent and that with increasing concentrations and volumes, healing was delayed. The time course and histopathology induced by intradermal or perivascular injection of various doses of either doxorubicin or caustic chemicals has also been studied in hairy outbred and hairless inbred mice.[13] The latter strain was found to be intrinsically more sensitive to doxorubicin-induced toxic effects, particularly as far as perivascular administration is concerned. Long-lasting lesions and, in a few cases, systemic involvement, were observed with doxorubicin, in contrast to caustic chemicals which induced necrotic foci that rapidly regressed in both strains of mice.

Although rats and other loose-skinned animals are used as experimental models, the similarity of pig's skin to that of humans has made porcine models preferred. In this study, we developed a porcine model of hard-to-heal wounds that limits their healing potential by forming a dead, mummified barrier around the wound edge, leaving a non-healing central wound bed, representative of hard-to-heal wounds in humans. This model then allowed us to evaluate the debriding efficacy of a novel bromelain-based enzymatic debriding agent in a hard-to-heal wound in an in vivo porcine model. We hypothesised that wound debridement would be achieved with fewer applications of the novel agent when compared with the commercially available collagenase product.

Methods

Animals

We used female domestic pigs (*Sus scrofa domestica*) of at least two months of age, weighing between 30–50kg at the start of the experiment. The animals were allowed to acclimate after their arrival. They were exposed to recurring cycles of light and darkness based on the presence of sunlight during the day. The animals were fed a standard pig diet twice a day with unlimited access to drinking water. The study was approved by the Institutional Animal Care and Research Committee and followed the National Research Council's Guide for the Care and Use of Laboratory Animals.

Test materials

Doxorubicin was supplied as a sterile 2mg/ml solution in normal saline (Parke-Davis, US) and further diluted to achieve various concentrations. Eschar-Ex-02 (MediWound, Israel) is a concentrate of proteolytic enzymes enriched in bromelain derived from the stems of pineapples. Collagenase (Santyl 250 unit/g,

Smith+Nephew Inc., US) is the only commercially available topical enzymatic debridement agent, which was used as the control.

Animal preparation

Animals were fasted overnight before initiation of the experiment and sedated with a combination of intramuscular xylazine (2mg/kg), ketamine (10mg/kg) and diazepam (5mg). General anaesthesia was obtained with inhalational 1–5% isoflurane in oxygen administered via a nose cone. The hair was removed with electric clippers and the skin was scrubbed with chlorhexidine (SeptalScrub, Teva Medical, Israel) and an iodine solution (Betadine, Purdue Products, US). The skin was then blotted dry with gauze.

Model establishment

On each of three animals, eight 3cm×3cm full-thickness wounds were created with a surgical scalpel, evenly distributed between the two sides of the vertebral column on the dorsum of the pigs. The wounds were digitally photographed and covered with a thin layer of petrolatum gauze and outer elastic adhesive dressing.

Various concentrations (0.25, 0.5 and 0.75mg/ml) and volumes (2.4 and 4.8ml per wound) of doxorubicin were injected intradermally into the periphery of the full thickness wounds one and six days after wound creation. A description of treatment assignments that takes into consideration even distribution of treatments between caudal wounds (located closer to the tail) and cephalic wounds (located closer to the head) is presented in Fig 1. During the first nine days after wound creation, the wounds were covered with a thin layer of petrolatum gauze. On days 9–10 the wounds were covered with a non-permeable layer of parafilm, and from days 11–20 the wounds were covered with dry gauze. Immediately before its removal, the dry gauze was made wet with saline to avoid its adherence to the wound since this may have resulted in additional mechanical wound debridement at the time of dressing change.

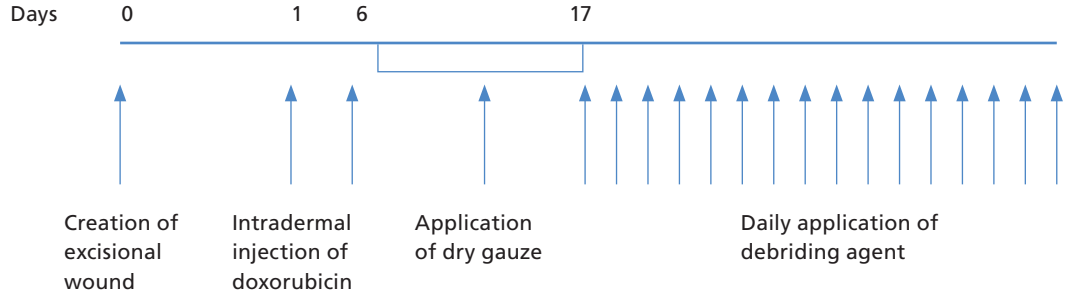
During the entire experiment the pigs were given intramuscular 4% Tolfedine 4mg/kg on a once-daily basis. On days zero and six, the pigs were also given intramuscular Tramadol 100 mg three times daily for 24 hours.

Assessment of eschar removal

In this phase of the study, to allow more distance between the wounds, smaller 1.5cm×1.5cm full-thickness excisional wounds were created using a scalpel on nine additional pigs. These nine pigs also received experimental treatments that were not part of the experiments reported in this manuscript. The volume of doxorubicin was adjusted to the reduced wound dimensions in a similar ratio; therefore 2.4ml of doxorubicin 0.75mg/ml was injected intradermally to the wound edges one and six days after wound creation as described above. During the first six days after wound creation, the wounds were covered with a thin layer of petrolatum-impregnated gauze. On days 6–17 the wounds were covered with dry gauze. Immediately prior to its removal, the dry gauze was made wet with saline to avoid its adherence to the wound since this may have resulted in additional mechanical wound debridement at the time of dressing change. Before and after each treatment all wounds were photographed with a digital camera.

We evaluated the number of treatments of various concentrations of EscharEx-02 (0, 0.1, 0.5, 2 and 5%) (n=7, 8, 8, 7, 2, respectively, total of 32 wounds) and collagenase (Santyl 250 unit/g) (n=11), the only commercially available enzymatic debriding agent, that was needed in order to achieve >95% eschar removal (as assessed clinically).[14] The treatments were applied for

Fig 2. Study design for assessing eschar removal



24 hours on a daily basis starting 17 days after wound creation (when the wound edges were completely necrotic and the centre of the wound was covered by slough) and continuing for up to 16 daily applications or until complete debridement (>95%) was achieved, whichever was sooner (Fig 2).

Data analysis

Binary data (percent complete eschar removal) were summarised as counts and percentages and compared with Chi-squared or Fisher exact tests. Continuous data (number of applications required to achieve complete eschar removal) were summarised as medians and interquartile ranges (IQR) and compared with the Kruskal–Wallis test with the Bonferroni correction for multiple tests. Repeated measures analysis of variance (ANOVA) was used to compare percent wounds with complete eschar removal over time. The unit of analysis was the wound, not the pig.

Results

Model development

As long as the wounds were covered with petrolatum gauze, they did not develop any apparent central eschar. When the wounds were covered with parafilm they developed an erythematous rash around their entire periphery (Fig 3a). When the wounds were covered with dry gauze, they developed an obvious central eschar (Fig 3b). The eschar that developed was composed of two distinct areas. A central area of eschar composed of exudate and materials secreted from the wound, and a surrounding area of full-thickness, doxorubicin-induced mummified necrosis at the wound edges.

The effects of intradermal injection of doxorubicin on skin necrosis and eschar formation were dose dependent. In all wounds where 2.4ml was injected intradermally to the skin surrounding the 3cm×3cm wounds, the necrosis was not homogenously dispersed in the wound periphery (Fig 4a, 4b, 4c). With the 4.8ml injection the necrosis was more evenly dispersed in the wound periphery, and the centre of the wound was completely covered with eschar (slough composed of materials secreted from the wound and bacteria) (Fig 4d, 4e, 4f). In the 0.25mg/ml concentration, the necrotic tissue was not very deep. With increasing concentrations, the eschar became larger and thicker. With the 4.8ml 0.75mg/ml concentration, the wound periphery was clearly necrotic and the centre of the wound contained a thick eschar (Fig 4f). While wounds which were not injected with doxorubicin were closed within a mean (standard deviation (SD)) of 25.7 (4.7) days, none of the wounds which were injected with doxorubicin completely closed during the 46 days of the study. We also found that wound location had an important impact on the rate of healing, where caudal wounds (located closer to the tail) healed more slowly and cephalic wounds (located closer to the head) healed faster (data not shown).

Fig 3. A full-thickness excisional wound 10 days after creation; erythema in the healthy skin surrounding the wound is distinct **(a)**. A wound 18 days after creation, covered by gauze; two distinct eschar types are apparent: in the centre, a slough composed of materials secreted from the wound, and in the periphery, completely necrotic skin where doxorubicin was injected; the healthy skin surrounding the wound (periwound) shows no apparent irritation **(b)**

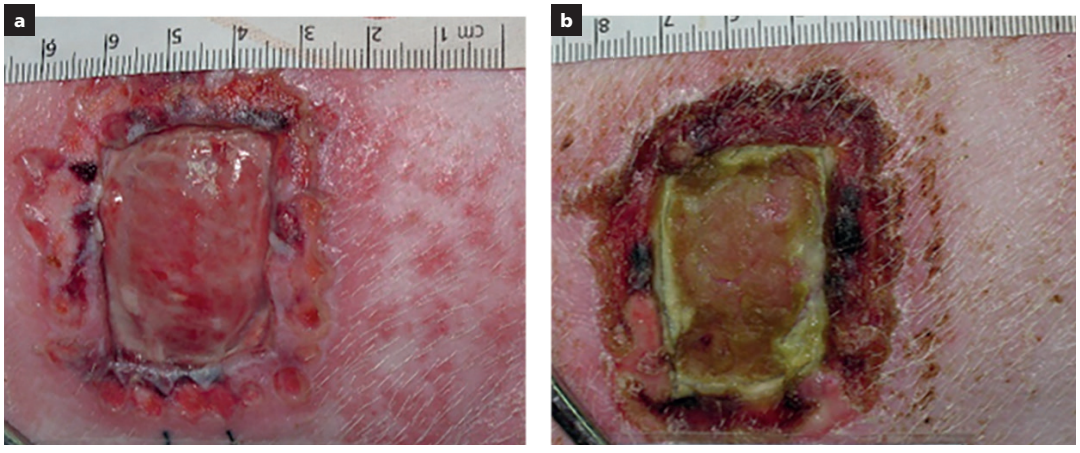
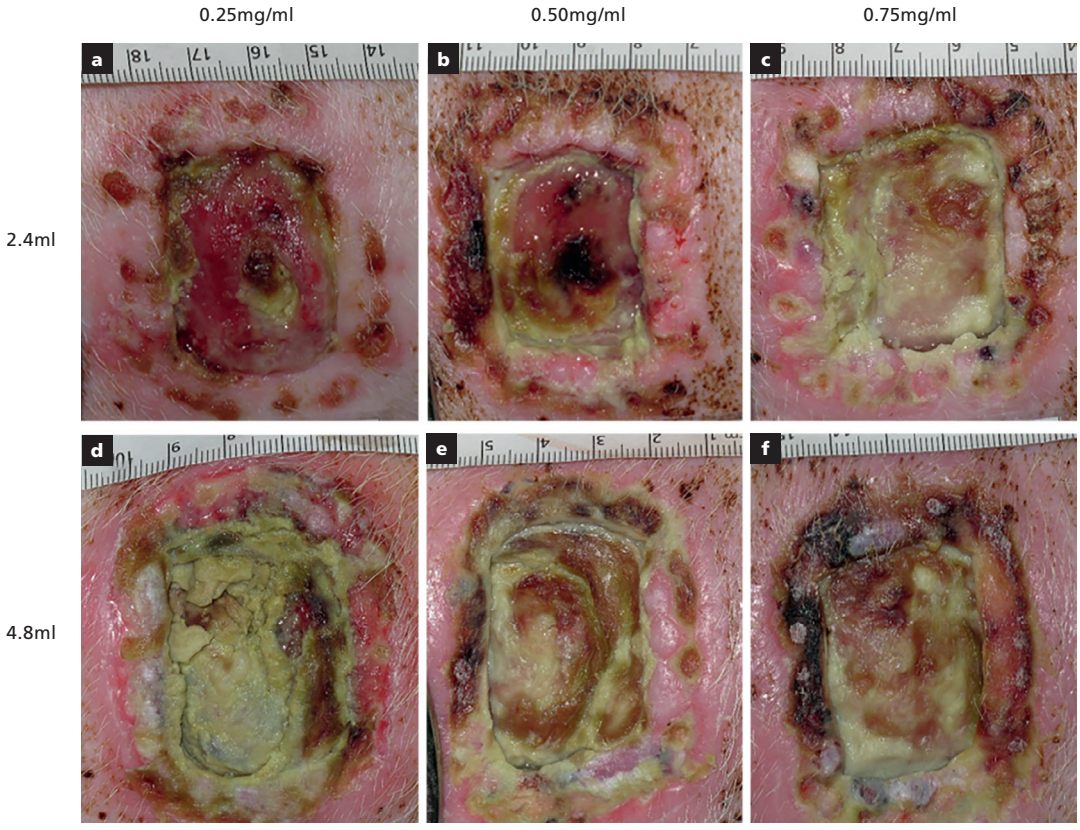


Fig 4. Representative 3cm×3cm wounds with varying concentrations and volumes of doxorubicin injection. With increasing volumes and concentrations, the necrotic tissue at the wound periphery is more homogenously dispersed and the central eschar is thicker: 2.4ml of 0.25mg/ml doxorubicin **(a)**; 2.4ml of 0.50mg/ml doxorubicin **(b)**; 2.4ml of 0.75mg/ml doxorubicin **(c)**; 4.8ml of 0.25mg/ml doxorubicin **(d)**; 4.8ml of 0.50mg/ml doxorubicin **(e)**; 4.8ml of 0.75mg/ml doxorubicin **(f)**



Histological observation of punch biopsies taken from the wounds and stained with haematoxylin and eosin (H&E) confirmed the clinical observation. The periphery of the wound demonstrated full-thickness mummification of the skin with underlying deep granulating tissue (Fig 5a). The centre of the wound demonstrated pauci-cellular slough on top of deep granulating tissue devoid of epidermis and dermis (Fig 5b).

Assessment of eschar removal

The model described above was used to assess a new enzymatic product developed for debridement of hard-to-heal wounds, EscharEx-02, and to compare its debridement efficacy with that of collagenase, the only commercially available enzymatic debridement product.

Since the eschar in hard-to-heal wounds (such as diabetic foot ulcers and venous leg ulcers) more closely resembles the eschar formed in the centre of the wound in our model, the primary outcome measured was time to complete debridement, i.e., clinical assessment of at least 95% eschar removal from the centre of the wound.

The efficacy of eschar removal was assessed using different strengths of EscharEx-02. In all treated wounds and at all concentrations, the wound eschar was completely removed within a maximum of 10 24-hour applications of EscharEx-02. A trend towards dose-dependency was observed in time to complete debridement (Fig 6).

The number of 24-hour daily applications needed to achieve >95% removal of the wound eschar was measured for all concentrations of EscharEx-02 and for collagenase (Fig 6). After 10 applications (the maximum

Fig 5. Representative micrograph of haematoxylin and eosin (H&E)-stained tissue taken from the wound. Wound periphery; mummified skin including deep dermis and underlying fat (*infiltrate rich in inflammatory cells, arrow denotes deep granulation tissue) **(a)**; wound centre (**oedematous granulation tissue, arrow denotes deep granulation tissue) **(b)**

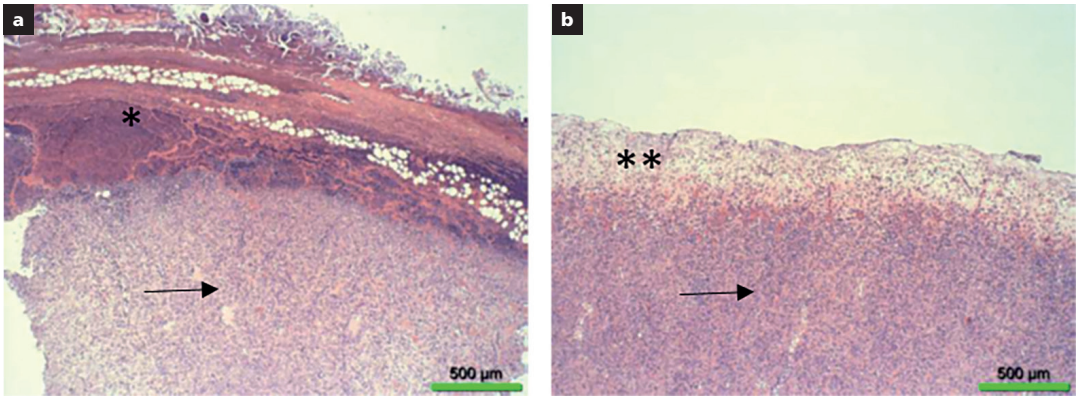
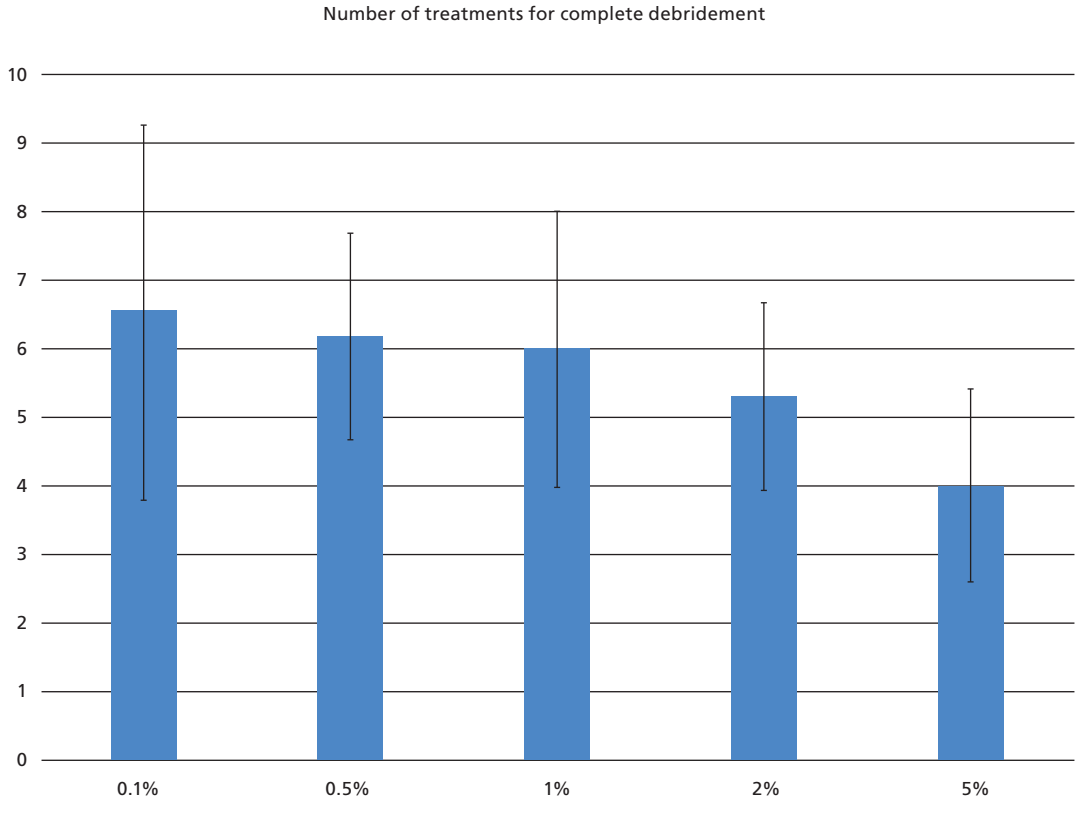


Fig 6. Number of daily applications needed to achieve >95% eschar removal in increasing EscharEx-02 doses. Dose dependency is observed



number of applications needed to achieve complete debridement with EscharEx-02 at all concentrations tested) none of the wounds treated with collagenase achieved >95% wound eschar removal. After 14 applications, only 45% of the wounds treated with collagenase achieved >95% eschar removal. After 16 applications (the maximum number of applications in the study), 82% of the wounds reached the endpoint of complete eschar removal. Fig 7 presents box-plots for the number of applications of EscharEx-02 (0.1–5.0%) or collagenase required to achieve complete eschar removal by treatment assignment. Pairwise comparisons between wounds treated with collagenase and each of the concentrations of EscharEx-02 demonstrated significantly fewer applications required with the EscharEx-02 (adjusted $p<0.05$ for all EscharEx-02 concentrations). Analysis of variance comparing mean number of applications required to achieve complete eschar removal also showed a significant difference in means ($p<0.001$); post hoc analysis using Tukey's B showed that all EscharEx-02 concentrations were similar, and that the EscharEx-02 groups were different than the collagenase group.

Pair-wise comparisons between wounds treated with collagenase and the various concentrations of EscharEx-02 demonstrated significantly higher percentages of complete eschar removal with the EscharEx-02 preparation for all timepoints except days 1 and 2, because no wounds in any group were debrided with only 1 or 2 applications (Table 1). Fig 8 shows the percentages of wounds with complete eschar removal

Fig 7. Box-plots for number of applications of EscharEx-02 (0.1–5.0%) or collagenase (COL) required to achieve complete eschar removal. Centre bars represent the median, the boxes represent the interquartile range and the outer bars are the outliers

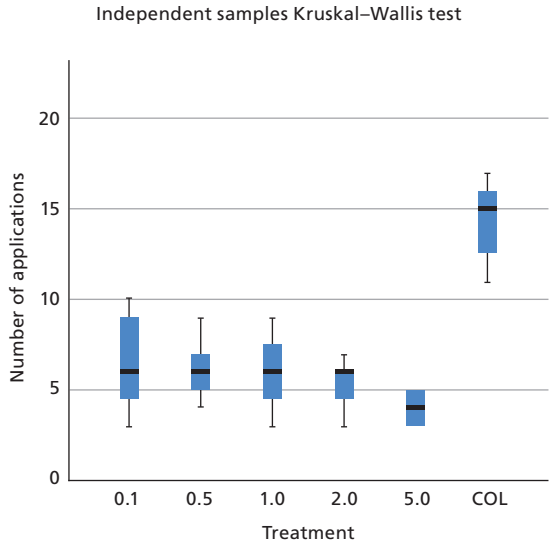
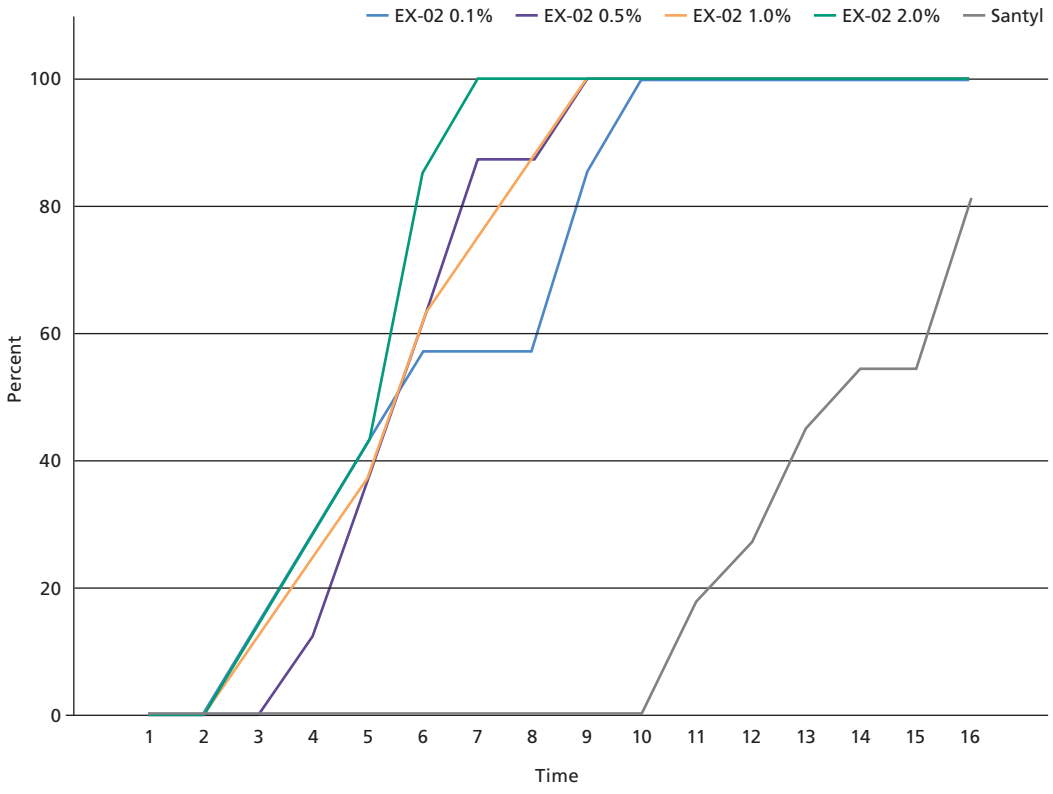


Table 1. Percentage eschar removal for collagenase and the four EscharEX-02 (EX) groups; values in parentheses are p values for the pairwise comparisons between Santyl and each of the EX groups. Numbers in square brackets are standard errors of the proportions in percent using the continuity correction

Day	Collagenase (n=11)	EX 0.1% (n=7)	EX 0.5% (n=8)	EX 1.0% (n=8)	EX 2.0% (n=7)
3	0% [5]	14% [20] (0.39)	13% [18] (0.42)	13% [18] (0.42)	0 [7]
4	0% [5]	29% [24] (0.14)	13% [18] (0.42)	25% [22] (0.16)	14% [20] (0.39)
5	0% [5]	43% [26] (0.04)	38% [23] (0.06)	38% [23] (0.06)	43% [26] (0.04)
6	0% [5]	57% [26] (0.01)	63% [23] (0.005)	63% [23] (0.005)	86% [20] (<0.001)
7	0% [5]	57% [26] (0.01)	88% [18] (<0.001)	75% [22] (0.001)	100% [7] (<0.001)
8	0% [5]	57% [26] (0.01)	88% [18] (<0.001)	88% [18] (<0.001)	100% [7] (<0.001)
9	0% [5]	86% [20] (<0.001)	100% [6] (<0.001)	100% [6] (<0.001)	100% [7] (<0.001)
10	0% [5]	100% [7] (<0.001)	100%[6] (<0.001)	100% [6] (<0.001)	100% [7] (<0.001)
11	18% [16]	100% [7] (0.002)	100% [6]	100% [6] (0.001)	100% [7] (0.002)
12	27% [18]	100% [7] (0.004)	100% [6] (0.003)	100% [6] (0.003)	100% [7] (0.004)
13	45% [20]	100% [7] (0.04)	100% [6] (0.02)	100% [6] (0.02)	100% [7] (0.04)
14	55% [20]	100% [7] (0.10)	100% [6] (0.05)	100% [6] (0.05)	100% [7] (0.10)
15	55% [20]	100% [7] (0.10)	100% [6] (0.05)	100% [6] (0.05)	100% [7] (0.10)
16	82% [16]	100% [7] (0.50)	100% [6] (0.49)	100% [6] (0.49)	100% [7] (0.50)

Fig 8. Percentages of wounds with complete eschar removal over time. The units on the time axis represent the number of applications. Repeated measures analysis of variance demonstrated significantly delayed eschar removal with collagenase (Santyl) compared with all concentrations of EscharEx-02 (EX-02).



by number of applications. Repeated measures analysis of variance demonstrated significantly delayed achievement of complete eschar removal with collagenase versus all concentrations of EscharEx-02.

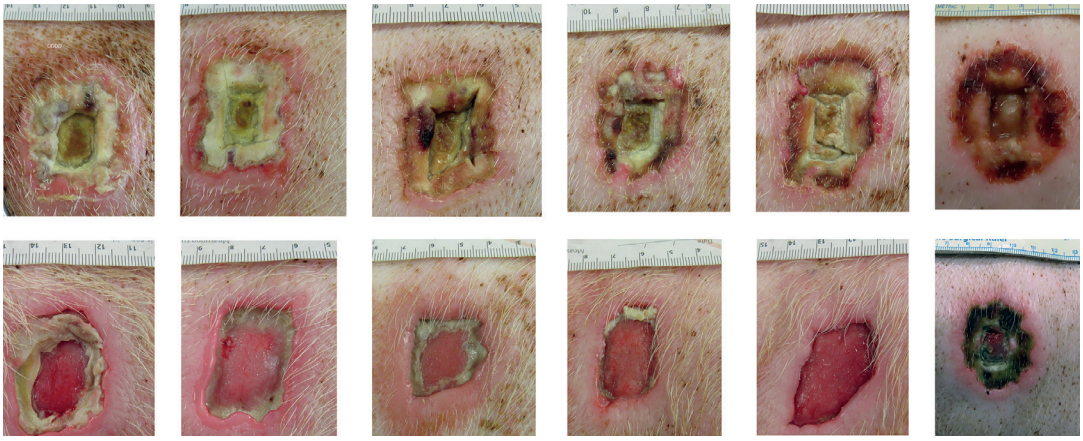
Complete debridement was defined in our model as >95% eschar removal from the centre of the wound. It is interesting to note that while collagenase did not remove the doxorubicin-induced necrotic tissue that was formed at the periphery of the wound where the doxorubicin was injected, treatment with EscharEx-02 dissolved this necrotic and mummified tissue at the wound periphery in a dose dependent manner. Fig 9 shows representative paired images of the same wounds before and after 10 treatments with different doses of EscharEx-02 as well as with collagenase.

Discussion

Our results demonstrate that a central eschar, similar to that seen in human hard-to-heal wound beds, can be consistently formed in a porcine model of delayed healing of full thickness excisions injected intradermally with doxorubicin. We further demonstrated that a bromelain-enriched enzymatic debriding agent (EscharEx-02) is effective at debriding the central eschars in this model. We found that EscharEx-02 is more effective in debriding the eschar in this model than Santyl, a commercially available enzymatic debridement agent based on collagenase. While there is no validated experimental animal model that fully replicates all of the phases and characteristics of hard-to-heal wounds in humans, our proposed model may by useful for studying the early and critical phase of hard-to-heal wound debridement or eschar removal.

We have previously described a contaminated ischaemic wound model in swine in which thick eschars are formed by cutting off the blood supply to the skin by 'sandwiching' the skin between two 'O' rings that are compressed together.[15] Tangential excision of the

Fig 9. Representative paired images from the same wounds at baseline (before application of the debriding agent in the upper row) and after application of the debriding agent (lower row). The columns from left to right were treated with EscharEx-02 0.1%, EscharEx-02 0.5%, EscharEx-02 1.0%, EscharEx-02 2.0%, EscharEx-02 5.0% and collagenase (the far right column).



superficial layer of the eschar is then followed by bacterial contamination leading to infected eschars. These eschars in pigs eventually slough off spontaneously and the wounds heal by granulation tissue formation and re-epithelialisation within ~2 weeks. The current doxorubicin model has several major advantages over our previous model. First, creation of necrotic wounds using intradermal injection of doxorubicin is far less technically challenging than the previous model. Second, the type of central eschar formed is more similar to the eschar in wounds such as venous leg ulcers and diabetic foot ulcers characterised by exudate, slough and necrosis. Third, the doxorubicin-induced eschars in the current model remain for a much longer period, allowing the formation of the central hard-to-heal wound and longer-term assessment of the wound and its debridement. Other methods aimed at simulating hard-to-heal wounds in animals have included induction of diabetes, creation of ischaemic skin flaps, administration of systemic corticosteroids and local radiation. However, all of these methods have their own limitations.[4]

Wound debridement is essential to remove any nonviable or devitalised tissue or materials in order to initiate wound healing.[2] Various methods of debridement have been proposed for necrotic wounds or ulcers, including mechanical, autolytic, biologic and enzymatic.[3] Surgical debridement, although effective, is traumatic and painful. It is also less selective than other methods of debridement. Wound irrigation, or hydrotherapy, is another method of mechanical debridement. More recently, ultrasound and laser have also been used to debride necrotic ulcers.[16,17] However, their use requires special facilities and personnel and is costly. Maggots have also been used for debriding necrotic wounds; however, their use has mostly fallen out of favour.[18] Autolytic debridement is based on endogenous proteolytic enzymes derived from bacterial contaminants in the moist wound environment. This process is very slow and exposes patients to local wound infection and sepsis. Enzymatic debridement using a variety of enzymatic agents has also been described. Currently, the only US Food and Drug Administration (FDA)-approved agent for over 50 years is collagenase which is slow acting (compared with EscharEx-02 in this study) limiting its usefulness.[19] The enzymatic agent evaluated in this study is a bromelain-enriched mixture of proteolytic enzymes derived from the stems of pineapples. We have previously shown that a similar formulation is effective at debriding necrotic wounds in our porcine model of contaminated ischaemic wounds.[15] Preliminary data in 24 patients have also demonstrated the ability of a similar formulation to debride hard-to-heal wounds of various aetiologies when applied daily for four hours at a time up to 10 times.[20] The formulation used in this study is undergoing phase two clinical trials in various countries worldwide (Clinicaltrials.gov NCT03588130) in which the

debriding agent is applied and left in place for 24 hours at a time. Because topical application may result in moderate yet temporary pain, the wound bed can be treated with a topical local anaesthetic supplemented by an oral non-steroidal anti-inflammatory agent when necessary.

Limitations

Our study has several limitations. First, the sample size for each strength of EscharEx-02 was relatively small. Therefore, we used a multifactorial experimental design to observe dose dependency. Second, similar as they may be, eschars formed in this model are probably not identical to eschars seen in human hard-to-heal wounds. Third, despite similarities between human and porcine skin anatomy and physiology, the results obtained in pigs may not generalise to humans. Finally, we did not have control wounds that were not treated at all or treated with a hydrogel in the eschar removal experiment. However, this was outside the scope of the study since the objective was to compare the two enzymatic debridement formulas.

Conclusion

In summary, we describe a novel porcine model in which eschars can be formed by injecting the edges of full-thickness excisional wounds with intradermal doxorubicin. We also demonstrate that a novel bromelain-based enzymatic agent, EscharEX-02, is more effective at debriding the eschars than the only commercially available collagenase, Santyl. Results from ongoing clinical trials with the novel agent will further inform us of its potential use in humans with hard-to-heal wounds. **JWC**

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