BMC Research Notes

RESEARCH NOTE

Open Access

An optimized mouse model of *Staphylococcus aureus* infected diabetic ulcers

Ana Isabel MENDES[1,2], Maria João PEIXOTO[1,2], Alexandra Pinto MARQUES[2,3], Jorge PEDROSA[1,2] and Alexandra Gabriel FRAGA[1,2*]

Abstract

Objective: Diabetic foot infection (DFI) represents a major healthcare burden, for which treatment is challenging owing to the pathophysiological alterations intrinsic to diabetes and the alarming increase of antimicrobial resistance. Novel therapies targeting DFI are therefore a pressing research need for which proper models of disease are required.

Results: Here, we present an optimized diabetic mouse model of methicillin-resistant *Staphylococcus aureus* (MRSA)-infected wounds, that resemble key features of DFI, such as pathogen invasion through wound bed and surrounding tissue, necrosis, persistent inflammation and impaired wound healing. Thus, in a time-efficient manner and using simple techniques, this model represents a suitable approach for studying emerging therapies targeting DFI caused by MRSA.

Keywords: Chronic wounds, Diabetic mouse model, Impaired wound healing, Inflammation, MRSA infection

Introduction

Management of chronic ulcerative wounds is a critical worldwide healthcare challenge, associated with a high risk of morbidity and mortality [1, 2]. Diabetes is an important predisposition factor for skin ulceration, particularly on the foot, which is often complicated by infection [3, 4]. Several pathogens can be found in diabetic foot infection (DFI), but *Staphylococcus aureus* is the most common [5–7]. *S. aureus* typically forms biofilms, evading the activity of both the host immune system and antibiotics, hampering current treatment strategies [6, 8–10]. The lack of effective treatment is further aggravated, when considering the alarming increase of methicillin-resistant *S. aureus* (MRSA) prevalence in DFI [7, 8]. Thus, the development of new therapeutic strategies for DFI is critical, for which appropriate and reliable models are urgently required. In this regard, this study developed an optimized protocol for generating standardized MRSA-infected wounds in a diabetic mouse model of impaired healing that mimics the main hallmark features of DFI, including continuous necrosis and inflammation associated with invasive infection of the wound bed, and can be easily employed to test novel therapies targeting DFI.

Main text

Methods

Animal experimentation was performed at the Life and Health Sciences Research Institute at the University of Minho, in accordance with the Directive 2010/63/EU, and approved by Institutional Animal Care and Use Committee of University of Minho. 8–12-week-old male C57BL/6 mice (Charles River Laboratories) were housed under specific pathogen-free condition with food and water ad libitum and acclimatized for 1 week before the experiment. Mice (n = 14) were equally and randomly divided in two groups corresponding to established endpoints of 2- and 9-days post-infection (dpi). Randomization was performed using randomize function of

*Correspondence: d8529@med.uminho.pt

[1] School of Medicine, Life and Health Sciences Research Institute (ICVS), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal
Full list of author information is available at the end of the article

Microsoft® Excel®. Humane endpoints were followed as described on Additional file 1: Table S1.

Diabetes induction

Type 1 diabetes mellitus (T1DM) was chemically induced, as previously described [11], administering 50 mg/kg of streptozotocin (STZ) (Merck KGaA, Germany) for 5 consecutive days. Blood glucose levels were measured 9 days after STZ treatment, using a monitor glucose device. Levels of blood glucose higher than 150 mg/dL were considered hyperglycemic. Mice were observed for signs of polydipsia and polyuria throughout the experimental period.

Dorsal fur depilation

On the day before surgery, dorsal fur of mice was shaved, using a hair clipper followed by depilatory cream for 1 min. Cream was further removed by wiping the skin with cotton soaked in warm water.

Inoculation of polycarbonate membranes

Briefly, 0.2 µm pore size polycarbonate membranes (Merck KGaA, Germany) were cut in 5-mm diameter discs, sterilized on both sides by UV light for 30 min and then placed on mannitol salt agar (MSA). A bacterial suspension of 10^8 colony forming units (CFU)/ml of *S. aureus* Rosenbach (ATCC BAA 2313) was prepared using isolated colonies previously grown on MSA, that were resuspended in saline and further inoculated on the membranes (10^2 CFU/membrane). Inoculated membranes were incubated overnight at 37 °C to grow a biofilm (attaining approximately 10^9 CFU/membrane).

Excisional wounding and infection

Mice were intraperitoneally injected with anesthetics (75 mg/kg ketamine and 1 mg/kg medetomidine) and analgesic (0.1 mg/kg buprenorphine). Two symmetrical full-thickness excisional wounds were created using a 5-mm diameter punch biopsy, by placing mice on their side, pulling the dorsal skin and perforating through the folded skin (Fig. 1A). To minimize wound contraction, a silicone splint ring (15-mm external and 6-mm internal diameter) was positioned around each wound using cyanoacrylate glue and secured with four interrupted sutures of 5/0 nylon (Fig. 1B). During the procedure, wounds were maintained hydrated with saline.

Wounds were infected by placing the *S. aureus*-inoculated polycarbonate membrane face down for direct contact of biofilm with the wound bed (Fig. 1C). Finally, wounds were covered with Durapore™ self-adhering bandage (3 M, USA) (Fig. 1D). Mice received 1 mg/kg atipamezole intramuscularly to revert the effect of anesthesia and were then placed under a warming lamp until full recovery. For postoperative pain relief, analgesia was administered subcutaneously with maximal intervals of 12 h during the two following days. Mice also received a vitamin supplementation (Duphalyte®) by subcutaneous injection, to avoid massive weight loss and dehydration.

Two days after wounding/infection, a biofilm covering the wounds was observed (Fig. 1E). At this point, mice received a light sedative (7.5 mg/kg ketamine and 1 mg/kg medetomidine, intraperitoneally) to remove the polycarbonate membranes. Topical treatment can be applied at this point, if desired. A sterile transparent semi-occlusive dressing Tegaderm (3 M, USA) was then applied covering wounds and splints, followed by an Omnifix elastic bandage (Hartmann) (Fig. 1F).

Wound tissue analysis

To assess the validity of the proposed model, wound tissue was collected for microbiological and histological analysis at 2- and 9-dpi. Mice were sacrificed with an overdose of isoflurane, and dressings and splints were carefully removed. Using a scalpel blade, wounds and surrounding skin tissue were harvested. One wound was used for bacterial burden quantification, while the other was dissected for histological analysis. For bacterial quantification, wound tissue was minced, tenfold serially diluted in sterile saline and cultured on MSA at 37 °C/24 h. The number of viable bacteria was expressed as Log_{10}CFU/wound. For histological analysis, tissue was divided across the wound center and immersed in neutral buffered formaldehyde (4%, w/v), embedded in paraffin, and then sectioned in samples of 4-µm thickness for staining with Hematoxylin and Eosin (H&E) and Gram.

Statistical analysis

Data were reported as mean ± standard deviation. Differences were assessed through Student's t-test, using Graphpad Prism 7 (Graphpad Software Inc., CA). Statistical significance was set at $p < 0.05$.

Results

The implementation of diabetes was achieved with STZ by damaging insulin producing β cells of pancreatic islets. All animals were considered diabetic, showing blood glucose levels of 312.4 ± 90.8 mg/dL (Fig. 2A), excessive water consumption and urine production.

Regarding the infection of inflicted wounds, a biofilm covering the wound bed was clearly identified both macroscopically (Fig. 1E) and microscopically (Fig. 2C). The biofilm formation was identical in both wounds and among animals. At 2-dpi, wounds bacterial burden reached a mean log_{10}CFU of 8.32 that significantly increased to 9.14 at 9-dpi (Fig. 2B). Histological analysis revealed that MRSA was present not

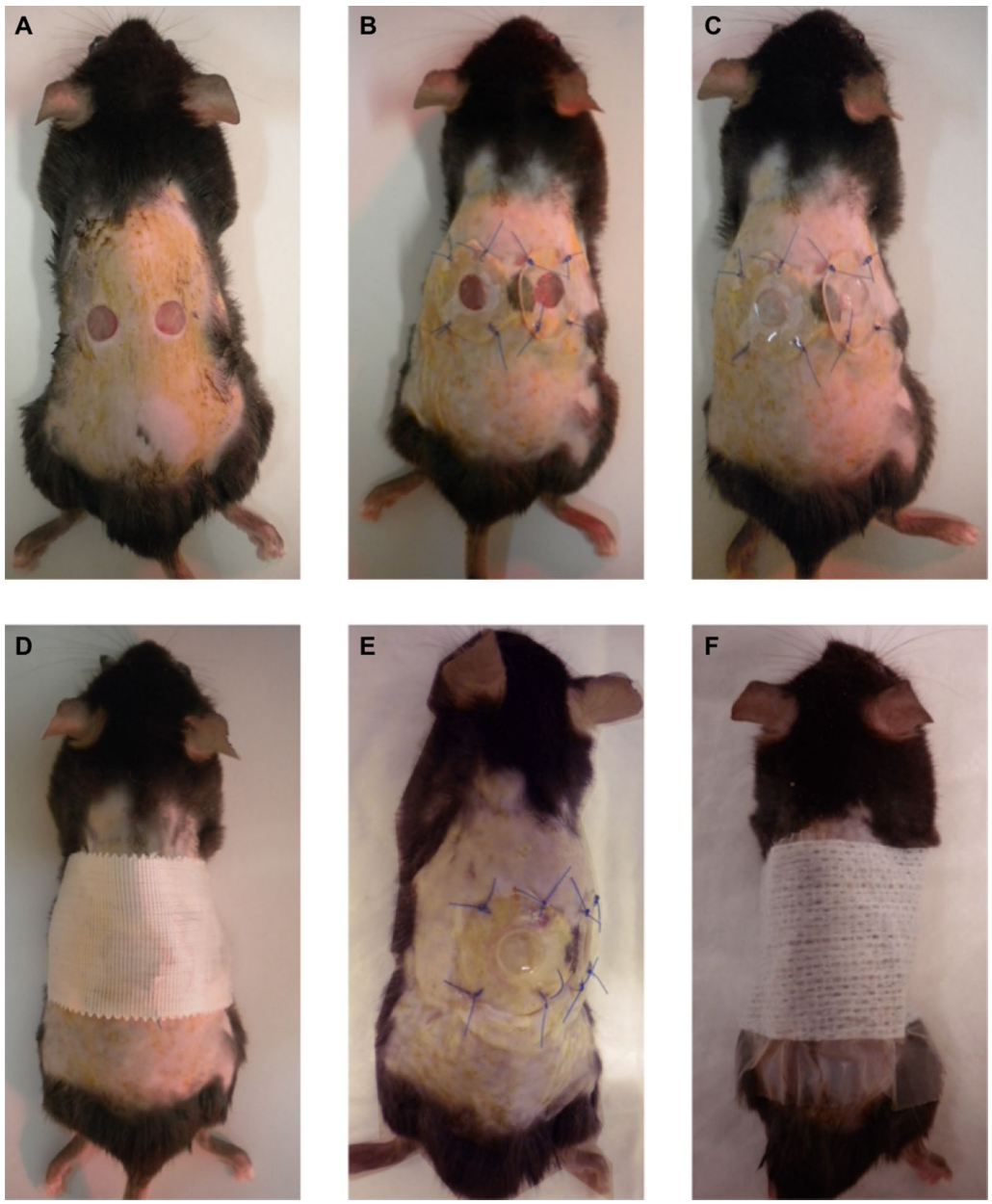

Fig. 1 Illustration of the protocol of full-thickness excisional wounding, infection and dressing: **A** creation of two symmetrical full-thickness wounds using a punch biopsy; **B** splinted wounds with silicone rings secured with cyanoacrylate glue and four interrupted sutures; **C** wound's infection with *S. aureus*-inoculated polycarbonate membranes; **D** wound's covering with self-adhering bandage; **E** representative photo of biofilm covering the wound 2 dpi; **F** wound dressing with a sterile transparent semi-occlusive dressing followed by an elastic bandage, after polycarbonate membranes removal

only on the wound surface but was also able to spread into the surrounding non-wounded deep tissue. Furthermore, infection led to necrosis and instigated the infiltration of inflammatory cells in the vicinity of the areas of bacterial accumulation (Fig. 2C). Wounds remained open over the experimental period, without signs of fibroplasia or granulation tissue formation, and revealed an identical pattern of inflammation and infection along different sections of wound area. It is noteworthy that two animals from the 9-dpi experimental group succumbed before the established endpoint.

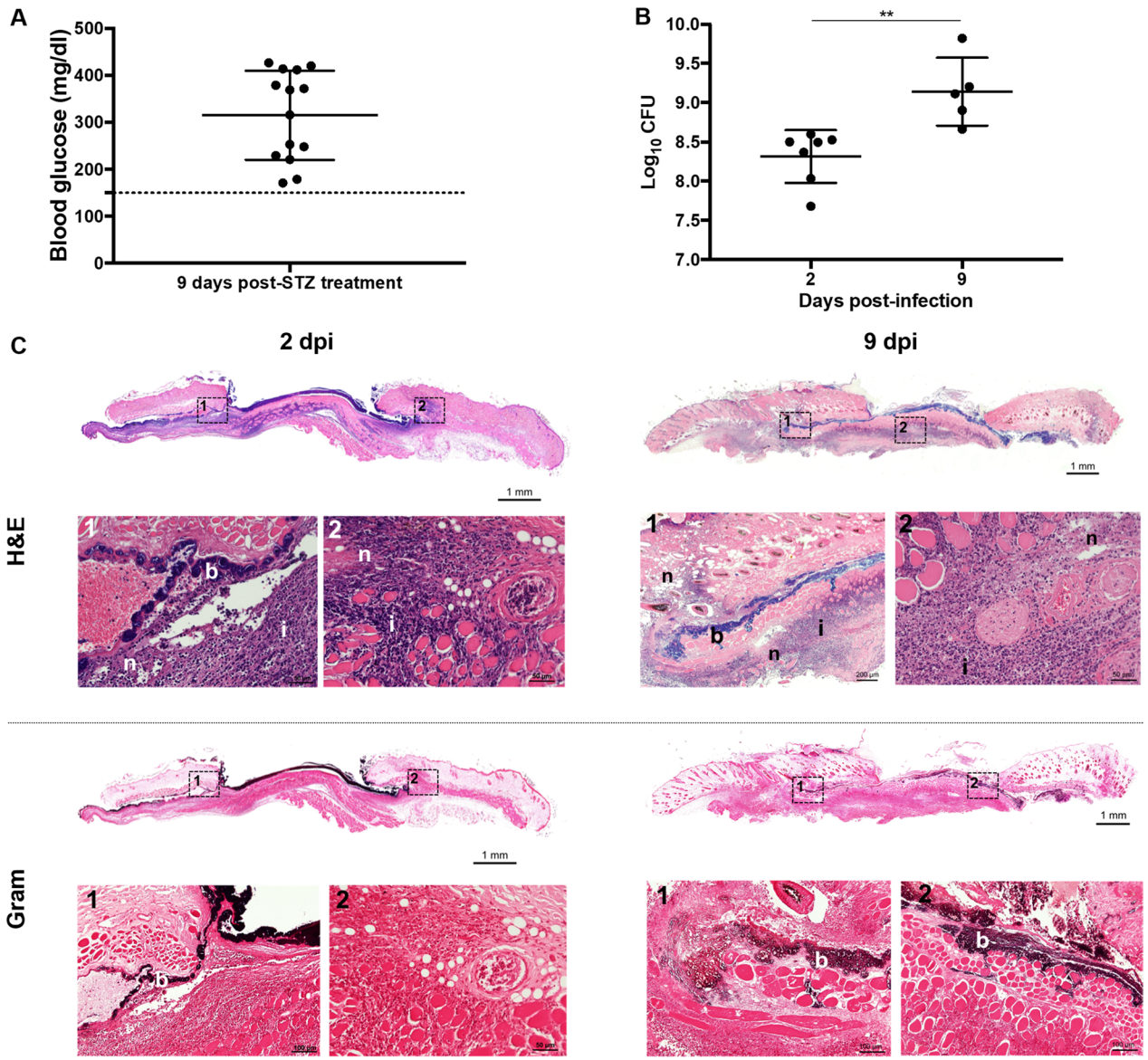

Fig. 2 Representative outcomes of the protocol of MRSA-infected wounds in a diabetic mouse model: **A** Blood glucose levels (mg/dL) after 9 days of STZ treatment; **B** bacterial burden of wounds at 2- and 9-dpi; **C** H&E and Gram-stained sections of wounds at 2- and 9-dpi (b: bacteria; i: inflammatory immune cells; n: necrosis)

Discussion

This study reports an optimized protocol to obtain standardized MRSA-infected chronic wounds in a diabetic mouse model that mimics human DFI pathophysiology. Not only did mice reveal signs of T1DM, such as hyperglycemia, polydipsia and polyuria [11], but experimentally inflicted wounds in diabetic mice showed an effective infection by MRSA that triggered continuous necrosis and infiltration of immune cells. This arrested state of necrosis associated with an inflammatory microenvironment hindered healing progression, representing key hallmarks of wound chronicity [2, 4]. Ultimately, this model depicts critical features of the DFI microenvironment that previous animal models of infected wounds failed to validate, including: i) the ability of MRSA to penetrate and spread within the wound bed, rather than being restricted to the scab [12–15]; ii) minimizing primary skin contraction that naturally occurs in rodents, which allows a healing mechanism by re-epithelization [12, 14, 16, 17]; and iii) control over the causative agent and dose of infection, circumventing the lack of standardization observed in models of naturally infected

wounds that depend on housing conditions, source of animal colonies and host skin microbiome [18].

All techniques herein employed were simple, easily executed and optimized in terms of timing and number of interventions to minimize animal morbidity and mortality, although they should be expected, specially at later timepoints of infection. Importantly, the behavior and mobility of mice were not significantly impacted by dressings, that remained in place and were not removed by mice, even though they were caged in group.

Overall, this model offers a simple and suitable approach for studying emerging technologies of topical application for the treatment of MRSA-infected chronic wounds, that are difficult to test in the available animal models. Ultimately, it can be adapted to different diabetic animal models or to other pathophysiological conditions.

Limitations

Wound infection with a single bacterial specie constitutes a limitation of this study. It would be relevant to further apply this protocol using polymicrobial biofilms to evaluate the interspecies relationship on the wound healing process.

Abbreviations

DFI: Diabetic foot infection; MRSA: Methicillin-resistant *Staphylococcus aureus*; Dpi: Days post-infection; T1DM: Type 1 diabetes mellitus; STZ: Streptozotocin; MSA: Mannitol salt agar; CFU: Colony forming units; H&E: Hematoxylin and Eosin.

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s13104-022-06170-5.

Additional file 1: Table S1. Animal welfare scoresheet and humane endpoints.

Acknowledgements

Not applicable.

Author contributions

AM: conceptualization, data collection and interpretation, writing – original draft preparation, writing—review and editing. MJP: data collection, writing—review and editing. APM and JP: conceptualization, data interpretation, writing—review and editing. AGF. Conceptualization, data collection and interpretation, writing—review and editing. All authors read and approved the final manuscript.

Funding

Financial support was provided to AM through the PhD grant UMINHO/BD/53/2017 supported by the project NORTE-08-5369-FSE-000041, financed by Operational Program NORTE2020 and co-financed by the European Social Fund. Financial support was also provided by National funds, through the Foundation for Science and Technology (FCT)-project UIDB/50026/2020 and UIDP/50026/2020.

Availability of data and materials

The data generated and analyzed during the current study are available from the corresponding author on reasonable request.

Declarations

Ethical approval and consent to participate

Animal experimentation was performed in accordance with the Directive 2010/63/EU and approved by Institutional Animal Care and Use Committee of University of Minho.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

Author details

[1]School of Medicine, Life and Health Sciences Research Institute (ICVS), University of Minho, Campus de Gualtar, 4710-057 Braga, Portugal. [2]ICVS/3B's–PT Government Associate Laboratory, Braga, Guimarães, Portugal. [3]3B's Research Group, I3Bs – Research Institute on Biomaterials, Biodegradables and Biomimetics, Headquarters of the European Institute of Excellence On Tissue Engineering and Regenerative Medicine, University of Minho, AvePark, Zona Industrial da Gandra, 4805-017 Barco, Guimarães, Portugal.

Received: 10 May 2022 Accepted: 10 August 2022
Published online: 07 September 2022

References

1. Avishai E, Yeghiazaryan K, Golubnitschaja O. Impaired wound healing: facts and hypotheses for multi-professional considerations in predictive, preventive and personalised medicine. EPMA J. 2017;8(1):23–33. https://doi.org/10.1007/s13167-017-0081-y.
2. Atkin L, Bucko Z, Montero EC, et al. Implementing TIMERS: the race against hard-to-heal wounds. J Wound Care. 2019;28(3):S5–50. https://doi.org/10.12968/jowc.2019.28.Sup3a.S1.
3. Armstrong DG, Boulton AJM, Bus SA. Diabetic foot ulcers and their recurrence. N Engl J Med. 2017;376(24):2367–75. https://doi.org/10.1056/NEJMra1615439.
4. Patel S, Srivastava S, Singh MR, Singh D. Mechanistic insight into diabetic wounds: Pathogenesis, molecular targets and treatment strategies to pace wound healing. Biomed Pharmacother. 2019. https://doi.org/10.1016/j.biopha.2019.108615.
5. Lipsky BA, Aragon-Sanchez J, Diggle M, et al. IWGDF guidance on the diagnosis and management of foot infections in persons with diabetes. Diabetes Metab Res Rev. 2016;32:45–74. https://doi.org/10.1002/dmrr.2699.
6. Smith K, Collier A, Townsend EM, et al. One step closer to understanding the role of bacteria in diabetic foot ulcers: characterising the microbiome of ulcers. BMC Microbiol. 2016;16(12):54. https://doi.org/10.1186/s12866-016-0665-z.
7. Silva V, Almeida F, Carvalho JA, et al. Emergence of community-acquired methicillin-resistant staphylococcus aureus EMRSA-15 clone as the predominant cause of diabetic foot ulcer infections in Portugal. Eur J Clin Microbiol Infect Dis. 2020;39(1):179–86. https://doi.org/10.1007/s10096-019-03709-6.
8. Negut I, Grumezescu V, Grumezescu AM. Treatment strategies for infected wounds. Molecules. 2018;23(9):2392. https://doi.org/10.3390/molecules23092392.
9. Mottola C, Matias CS, Mendes JJ, et al. Susceptibility patterns of Staphylococcus aureus biofilms in diabetic foot infections. BMC Microbiol. 2016. https://doi.org/10.1186/s12866-016-0737-0.
10. Zhao G, Usui ML, Lippman SI, et al. Biofilms and inflammation in chronic wounds. Adv Wound Care. 2013;2(7):389–99. https://doi.org/10.1089/wound.2012.0381.

11. Wu KK, Huan Y. Streptozotocin-induced diabetic models in mice and rats. Curr Protoc Pharmacol. 2008. https://doi.org/10.1002/0471141755.ph0547s40.
12. Silva V, Peirone C, Capita R, et al. Topical application of ozonated oils for the treatment of MRSA skin infection in an animal model of infected ulcer. Biology. 2021;10(5):372. https://doi.org/10.3390/biology10050372.
13. Shi CM, Nakao H, Yamazaki M, Tsuboi R, Ogawa H. Mixture of sugar and povidone-iodine stimulates healing of MRSA-infected skin ulcers on db/db mice. Arch Dermatol Res. 2007;299(9):449–56. https://doi.org/10.1007/s00403-007-0776-3.
14. Guo Y, Ramos RI, Cho JS, Donegan NP, Cheung AL, Miller LS. In Vivo bioluminescence imaging to evaluate systemic and topical antibiotics against community-acquired methicillin-resistant staphylococcus aureus-infected skin wounds in mice. Antimicrob Agents Chemother. 2013;57(2):855–63. https://doi.org/10.1128/aac.01003-12.
15. Zhao G, Hochwalt PC, Usui ML, et al. Delayed wound healing in diabetic (db/db) mice with Pseudomonas aeruginosa biofilm challenge: a model for the study of chronic wounds. Wound Repair Regen. 2010;18(5):467–77. https://doi.org/10.1111/j.1524-475X.2010.00608.x.
16. He H, Xia DL, Chen YP, et al. Evaluation of a two-stage antibacterial hydrogel dressing for healing in an infected diabetic wound. J Biomed Mater Res B Appl Biomater. 2017;105(7):1808–17. https://doi.org/10.1002/jbm.b.33543.
17. Tong CY, Zhong XH, Yang YJ, et al. PB@PDA@Ag nanosystem for synergistically eradicating MRSA and accelerating diabetic wound healing assisted with laser irradiation. Biomaterials. 2020;243(14): 119936. https://doi.org/10.1016/j.biomaterials.2020.119936.
18. Kim JH, Martins-Green M. Protocol to create chronic wounds in diabetic mice. J Vis Exp. 2019. https://doi.org/10.3791/57656.

Publisher's Note