Author's response to reviews

Title: Enhanced Susceptibility to Infections in a Diabetic Wound Healing Model

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Author's response to reviews: see over
Dear Dr Marlee

We are writing to you on behalf of the first revision of our submitted manuscript entitled “Enhanced Susceptibility to Infections in a Diabetic Wound Healing Model” MS: 5453020121504648. The reviewers outlined some issues concerning our manuscript and we kindly resubmit our revised manuscript with the responses to the referee comments. All authors have read and approved this revised manuscript and transfer all copyright ownership of this manuscript to BMC in the event the work is published.

Please let me know if I can provide any additional information and I will be happy to get back to you immediately.

Once again, thank you for considering this manuscript.

Yours sincerely,

Tobias Hirsch, MD
Response to Reviewer 1

1. Why such high bacterial counts in diabetic animals, can the authors do PMN or myeloperoxidase staining of their wound sections to determine immune derangements?
The aim of the study was to establish a large-animal infected wound model in hyperglycaemic state. Thus, we did not investigate the immune reaction in the wound tissue in particular. Our scope was to quantify the bacterial burden and its properties over time. However, we agree with the reviewer we are planning to incorporate this in future studies.

2. There is no discussion of the limited importance of pathogenic effects of the author’s proposed primary pathogen, Staph Aureus
Inoculation with Staph aureus caused significant infection in the diabetic wound tissue. Thus, we conclude that our primary pathogen (Staph aureus) is of significant importance to the pathogenic effect, since the wound healing delay caused by the hyperglycemic metabolic state is further impaired when wounds were inoculated with Stap.h aureus.

3. What were the serum glucoses during the experimental period? How were they controlled?
This important point has been added to the revised manuscript. To keep the blood glucose concentration between 250 and 500 mg/dl, pigs received daily injections of 10 IU insulin/10IU NPH insulin (Humulin, Eli Lilly, IN) subcutaneously. Blood glucose was measured on a daily basis during the experiment.

4. Were there not differences between contraction rates of diabetic wound with or without Staph or are the numbers to small to note the differences? Similarly, reepithelialization rates in inoculated wound in non-diabetic wounds. They need to do a power analysis before making their conclusions of no difference.
There were no differences between contraction rates in inoculated and non-inoculated diabetic wounds. This aspect has been pointed out in more detail in the revised
manuscript: *S. aureus*-inoculated diabetic wounds showed less contraction (24 +/- 23\% wound contraction day 12) compared with non-inoculated diabetic wounds (37 +/- 10\% wound contraction day 12); however, there was no significant difference between groups. The power analysis showed a power of 8\% for this study. To generate a power of 90\% 889 wounds are needed. In our model, this means that we would need to include 63.5 pigs in our study. However, due to economical and ethical reasons, we were not able to include such a number of animals in our study.

Reepithelialization was assessed in all study groups. Inoculated non-diabetic wounds showed no significant difference compared with inoculated non-diabetic wounds. This might be due to the efficient host response towards bacterial infections. In non-diabetic organisms. No significant difference in non-diabetic wounds inoculated with *S. aureus* compared to non-inoculated wounds (87 +/- 22\% versus 97 +/- 5\%) could be detected (Fig. 4).

5. There is too much discussion of Staph in the INTRO considering its limited effects for the study

The aim of the study was to establish a preclinical large-animal model for *S. aureus* infected diabetic wound healing model. Thus, *S. aureus* as bacterial strain plays a key role in our study as well as in wound infection in general. Therefore it was important to us to give an overview of *S. aureus* in wound infections in the introduction of the manuscript. However we have shortened the discussion of *S. aureus* in the introduction according to the reviewers suggestion: As early as the late 1940s, hospitals in the UK and the USA reported that *S. aureus* infections were resistant to penicillin[15], and in the 1960s the first methicillin-resistant *S. aureus* (MRSA) was identified[16].

6. The discussion is mostly restating the authors findings rather than putting them into context.

We have shortened the sections dealing with our findings in the discussion. To attach more value to the discussion of the results: Bacterial concentrations were consistently higher in the wound fluid than in the wound tissue in both diabetic and non-diabetic animals (by 2.5 to 3 log), and strains found in the fluid sometimes differed from the strains found in the tissue (data not shown).
Diabetic wounds showed a significantly delayed reepithelialization compared to non-diabetic wounds. Inoculation with S. aureus caused a further delay in diabetic wound healing, confirming the presence of an apparent invasive sustained bacterial infection in the wound tissue. Delayed healing in wounds infected by S. aureus is probably also responsible for the lower (though statistically non-significant) contraction rate in S. aureus-inoculated wounds compared to non-inoculated controls in the same animal. In non-diabetic animals, bacterial inoculation caused only a non-significant delay in wound healing, confirming the previous finding that diabetic animals have higher susceptibility and experience more severe complications after bacterial challenge.
More information about the “diabetic state” of the animals would be desirable. We have included more information about the diabetic state of the animals in our revised manuscript: To keep the blood glucose concentration between 250 and 500 mg/dl, pigs received daily injections of 10 IU insulin/10IU NPH insulin (Humulin, Eli Lilly, IN) subcutaneously. Blood glucose was measured on a daily basis during the experiment.

Minor Essential Revisions:

It is not clear how many biopsies are taken per wound for bacterial quantification. Four biopsies were taken from each wound. We have clarified this important point in the materials and methods section of the revised manuscript: Biopsy specimens (3 mm punch-biopsy) for bacterial quantification in tissue were obtained on days 4, 8, (n=3 wounds for S. aureus-inoculated wounds) and day 12 (n=4 wounds for all groups with 4 punch biopsies per wound).

The data should be presented either in a table or in a figure not both (table 1 and figure 2, and table 2 and figure 3 present the same data). We have deleted the tables according to the reviewers suggestion.

The nonparametric test used to evaluate the data should be given. The n is very low. We have indicated the statistic analysis in the material and methods section in the revised manuscript.

When the data are not normally distributed the data can best be presented as median with the range. The meaning of the symbols used in figure 1 (+, *, #, §) should be explained.

We mentioned the statistical anlaysis used in the revised manuscript in the material and methods section: This study included a total of 56 wounds in 2 diabetic pigs and 2 non-diabetic pigs divided into 4 experimental groups:

A: Diabetic wounds treated with a sterile 0.9% saline solution (n=14) B: Diabetic wounds inoculated 2x10^8 CFU S. aureus (n=14) C: Non-diabetic wounds treated with
sterile 0.9% saline solution (n=14) D: Non-diabetic wounds inoculated with 2x10⁸ CFU S. aureus (n=14). Values are presented as means ± SE. Groups were compared using the independent t-test, and statistical calculations were performed with GraphPad Instat software (GraphPad Software, CA). A p-value <0.05 was considered statistically significant.

The meaning of the symbols used in figure 4 has been explained and added to the revised manuscript: * = p<0.05 S. aureus-noculated diabetic versus non-inoculated diabetic wounds; # = p<0.05 S. aureus-inoculated diabetic versus S. aureus-inoculated non-diabetic wounds; § = p< 0.001 S. aureus-inoculated diabetic wounds versus non-diabetic wounds.