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Quality Assessment of Tissue Specimens for Studies of Diabetic Foot Ulcers

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Abstract

Diabetic Foot Ulcers (DFUs) represent an important clinical problem resulting in significant morbidity and mortality. Ongoing translational research studies strive to better understand molecular/cellular basis of DFU pathology that may lead to identification of novel treatment protocols. Tissue at the non-healing wound edge has been identified as one of major contributors to the DFU pathophysiology that provides important tool for translational and clinical investigations. To evaluate quality of tissue specimens and their potential use we obtained 81 DFU specimens from 25 patients and performed histological analyses, immunohistochemistry and RNA quality assessments. We found that depth of the collected specimen is important determinant of research utility, and only specimens containing a full-thickness epidermis could be utilized for immunohistochemistry and RNA isolation. We showed that only two-thirds of collected specimens could be utilized in translational studies. This attrition rate is important for designs of future studies involving tissue specimen collection from DFU.

Keywords

diabetic foot ulcer; tissue collection; specimen; RNA; immunohistochemistry

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Stojadinovic O.: Performed research, designed research study, analysed data and wrote the manuscript. Landon J.N.: Performed research, analysed data and wrote the manuscript.Gordon K.A.: Performed research. Pastar I.: Performed research. Escandon J.: Collected tissue specimens. Vivas A.: Collected tissue specimens. Maderal A.D.: Collected tissue specimens and provided patient demographical data. Margolis D.J.: Critically revised manuscript. Kirsner R.S. Critically revised manuscript. Tomic-Canic M.: designed research study, analysed data and wrote the manuscript

Conflict of Interest:

Authors declare no conflict of interest.

Background

The development of DFUs is an important clinical problem which leads to significant morbidity and mortality (1, 2). Diabetic foot ulcers are responsible for more hospitalizations than any other complication of diabetes and are the leading cause of non-traumatic lower extremity amputations in the United States (3, 4). In fact, 12–24% of patients with DFUs will ultimately require an amputation (5), resulting in nearly 100,000 amputations in the United States yearly (6). Therefore, prompt and effective treatments for DFUs, as well as a better understanding of the pathophysiology are necessary to prevent these potentially devastating outcomes. Surgical debridement is a central component of standard of care of DFUs (6–8) and is meant to remove healing-impaired tissue, decrease bacterial bioburden, and, as a result, stimulate overall wound closure, while removing as little of healing competent skin as possible. The non-healing wound edge is an important contributor to the pathophysiology of DFUs and is often used as a valuable tissue source for research purposes (9–14). Tissue removed from the wound during debridement can also be valuable diagnostic and research source to verify pathology, asses prognosis and gain insights into DFUs molecular pathology, all of which ultimately leads to improved outcomes.

Questions Addressed

We aimed to validate tissue obtained from surgical debridement of DFUs for utilization in translational research studies in order to provide a method for objective criteria for specimen evaluation. Many ongoing translational research studies involve the cellular/molecular analyses of tissues, including validating therapy, biomarkers, understanding mechanisms that inhibiting healing or mechanisms of action of various therapies, all of which require the acquisition of tissues from patients. However, there is no consistent approach to evaluate specimens in standardized fashion.

Experimental Design

In a prospective study we collected wound edge tissue specimens from 25 DFUs patients during surgical debridement at the first presentation to the clinic and four weeks later. One to four specimens were obtained from each patient per debridement, resulting in a collection of 81 specimens. Demographic characteristics of patient population are presented in Supporting Information (Table S1). Histology, immunofluorescence staining and RNA isolation were performed using standard methods (see Supporting Information).

Results

To evaluate debrided tissue, each tissue specimen was processed for paraffin embedding, and stained with hematoxylin and eosin. Histopathology analysis showed variability among specimens dependent on the depth of debridement (Figure 1a). We identified three depth categories among the tissue specimens: *callus only; partial specimens*-containing callus and some epidermis; and *complete specimens*-containing callus, the full thickness epidermis and a portion of the dermis. Histological findings commonly present in DFU's, including a thickened, hyper and para-keratotic epidermis were observed (6, 15–17). When multiple specimens were obtained around wound perimeter they also contained three depth categories

indicating that multiple specimens obtained from the same wound should be analyzed separately (Figure 1b). We found that two thirds (54/81 e.g. 66%) of specimens were complete as defined by presence of the dermis and epidermis in the specimen, which is essential for studies involving wound edge biomarkers, as well as studies delineating potential molecular mechanisms involved in healing pathology (Figure 1c).

A molecular marker, c-myc, was previously shown to be present in a non-healing edge of a chronic wounds (16). To evaluate how variability of the specimen collection may influence potential biomarker assessment, we used immunohistochemistry. Specimens containing callus only and partial specimens did not yield useful data, since biomarker presence could not be fully analyzed or quantified. However, complete specimens, showed epidermal presence of biomarker, resulting in useful information (Figure 1a).

To further explore the utility of these specimens for RNA analysis, which is commonly done in conjunction with cellular analysis in tissue samples, we isolated RNA from DFU edge tissue. RNA quality was assessed using a Agilent Bioanalyzer. Out of 32 tissue specimens examined, only 16 showed high quality RNA, as defined by RNA Integrity Number (RIN) a standard for RNA quality assessment. These specimens had RIN>6 and were deemed as valuable for further molecular analysis. Interestingly, we found that the histological depth of the tissue adjacent to that used for procuring RNA correlated with the RIN value. A representative RIN analyses are shown in Figure 2 a–c. A poor RNA quality as exhibited by a RIN< 6 was obtained from 71% and 91% of tissue derived from wound edge specimens adjacent to specimens containing callus only or from partial specimens, respectively. On the other hand, only 7% of tissue obtained from specimens adjacent to histologically confirmed complete specimens contained poor quality RNA (Figure 2d). We conclude that there is a likelihood of obtaining high quality RNA from the complete tissue specimens.

Conclusion

Current trends in translational wound healing research involve the study of specimens obtained from non-healing edges of chronic wounds for various cellular and molecular analyses (9–12, 18–21). Additionally, new therapeutic strategies such as utilization of stem cells for wound healing disorders would also benefit from mechanistic studies using chronic wound specimens (22–25). All these studies are crucial for the development of new standardized treatment protocols to combat the significant morbidity and mortality associated with DFUs. Our laboratory and others have recognized the physiologically impaired non-healing edge as an integral component of chronic wound pathology and this has led to the discovery of several potential biomarkers currently under investigation (16, 18, 20). These biomarkers are activated in all layers of the wound edge epidermis, and therefore accurate detection of these markers may depend on acquisition of tissue specimens (16, 18, 20). However, obtaining complete specimen may be particularly challenging at the DFU's wound edge since variability in epidermal thickness exists and a thickened cornified layer may mask the viable tissue underneath.

The absence of a full-thickness epidermis precludes accurate immunohistochemistry analysis. Furthermore, RNA integrity from partial specimens was significantly reduced,

hindering the ability to perform PCR and gene expression analysis. We conclude that specimen collection from DFU edges is essential for detection of reliable biomarkers either by immunohistochemistry or RNA analyses. Furthermore, the interpretation of data acquired from collected incomplete tissue specimens can lead to incorrect conclusions, thus introducing potential confounds into the studies.

In this report, we performed a thorough histology/morphology assessment of collected DFU specimens and showed that two-thirds of specimens contained the entire epidermis. The integrity of RNA isolated appears to be dependent on depth and suggests that 1/3 of specimens may have lower RNA integrity and would not support major molecular studies, such as microarrays. In contrast, we found that RNA integrity is likely to be of high quality in complete specimens. Moreover, we found that cellular and molecular analyses are only feasible in complete specimens, suggesting that full thickness biopsies should be utilized in obtaining specimens from DFU's. This approach will not only lead to better quality of acquired specimens and improved clinical trial outcomes, but will also be beneficial for patients since it's been documented that biopsying the wound does not delay overall healing of the chronic wound patients (26). We concluded that not all routinely obtained debridement specimens are appropriate for research purposes. Even in a clinical trial setting with an experienced clinical research team a significant portion of specimens may not be appropriate. Research teams should consider the type of analyses to be performed in the laboratory from the acquired specimens and such experimental design should be incorporated into clinical protocols. Morphological evaluation of obtained specimens should be performed prior to cellular/molecular analyses. Finally, we recommend training of clinical research personal to awareness to and techniques in obtaining full-thickness epidermal biopsies. The proposed approach for obtaining and processing DFU tissue specimens would impact the field of wound healing as it would improve design of large scale clinical trials for testing novel diagnostic and therapeutic modalities.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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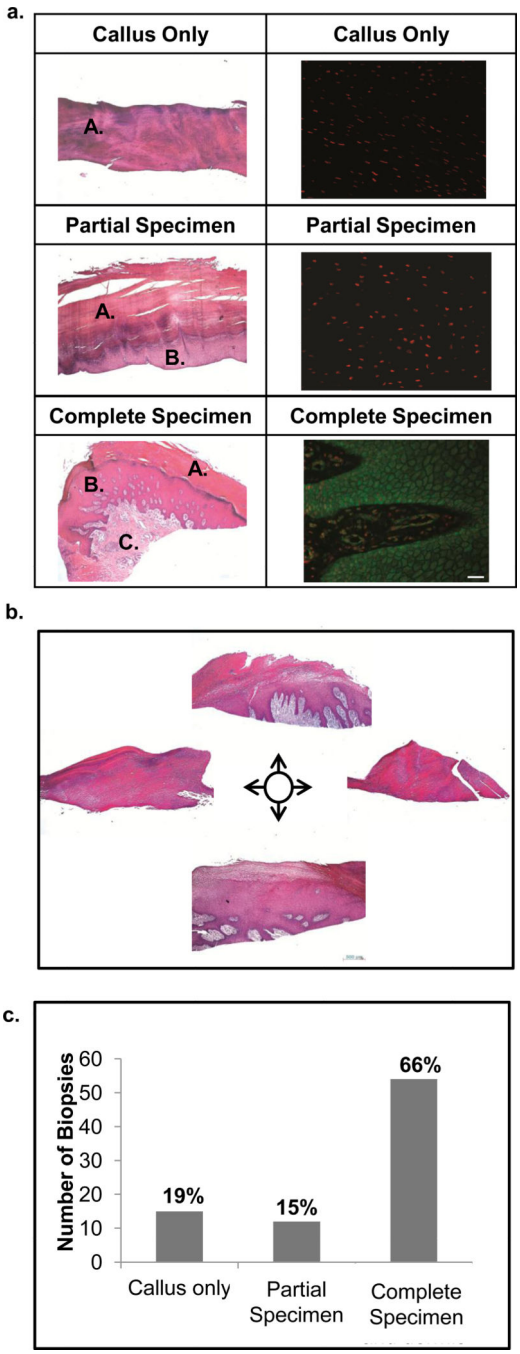

Figure 1. Tissue morphology of DFU specimens indicates histologic variability resulting from different depth

a. Representative histology (H&E) of specimens (left panel) and representative biomarker staining (right panel) collected form DFU patients is shown. A= Callus; B=epidermis; C= dermis. Green signal visualizes biomarker whereas red signal visualizies nuclei. Scale bar 200 μm. **b.** Representative histology (H&E) of specimens obtained from the different locations of the same wound are shown. **c.** One-third of the collected specimens did not contain adequate histology for further analysis. Graph summarizes distribution of 81

samples: 66% of specimens contained full thickness tissue specimen whereas 33% did not (19% of callus only and 15% of callus and partial thickness). Scale bar 500μm.

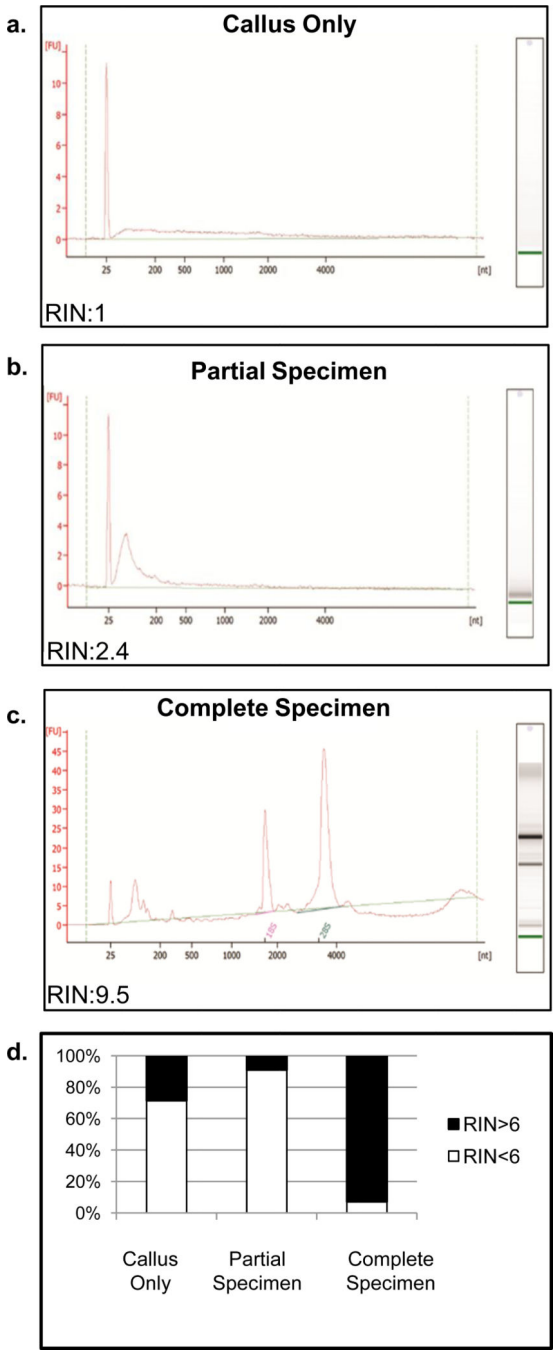

Figure 2. Specimen depth correlates with RNA quality

A representative electropherograms and RNA gel images are shown for all three categories of samples: complete specimen (**a**), partial specimen (**b**) and callus only (**c**). A graph summarizes distribution of RIN from specimens based on sample categories. High RNA integrity (RIN>6) is found predominantly in the full thickness specimens whereas the partial or callus only containing specimens showed RIN<6 **(d)**.