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A simple and improved method to determine cell viability in burn-injured tissue

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ABSTRACT

Background: Cell viability is paramount to wound healing in burn injury. Current methods to determine depth of burn injury in the research setting are based on the subjective visualization of cell viability using hematoxylin and eosin staining. The purpose of this study was to develop a simplified method of lactate dehydrogenase (LDH) staining to identify viable cells in frozen sections of human burn tissue that can be used in the research setting.

Materials and Methods: After surgical excision, human burn tissue was processed for histologic evaluation. Tissues were fixed and protected with sucrose incubation before cryopreservation. An LDH staining method was developed and evaluated for prolonged stain stability. To evaluate cellular viability in the tissues as demonstrated by enzymatic activity of LDH, digital images of tissue sections were obtained immediately after and 1 mo after staining.

Results: The cryopreserved sections of deep partial thickness human burn tissue revealed cellular viability throughout the tissue with the exception of the most superficial region of the tissue. Unlike the hematoxylin and eosin–stained sections, clear demarcation of cellular viability was evident in the LDH-stained sections.

Conclusions: Our simplified protocol identifies, without ambiguity, the viability of the cellular elements in deep partial thickness and full thickness burn injured tissue.

Burn injury depth determines the need for surgical intervention and is described in terms of the remaining dermis after injury. Clinically, partial thickness burns contain some viable dermal elements, whereas full thickness (FT) burns are devoid of all viable dermis. The regenerative capacity of skin is a function of the remaining dermal appendages containing the epidermal progenitor cells which serve to repopulate the wound for healing to occur.[1] Visual assessment is the most common method used clinically to determine the viability of tissue.[2] However, it has been reported that even experienced surgeons accurately assess tissue viability and thus the need for surgery in only 50% of the cases.[3] Furthermore, tissue containing a preponderance of viable cells is often removed using current surgical excision methods necessary to prepare the wound for skin grafting to prevent graft loss if nonviable tissue is left behind.[4] To determine the potential success or failure of burn care diagnostic and therapeutic innovations currently under development, we must perform a clear assessment of the wound bed to provide an adequate understanding of the baseline wound before application.

Studies in rat,[5,6] pig,[7] and humans[8] have used a hematoxylin and eosin (H&E) assay to assess cell viability in excised burn tissues. In addition, research on wound healing therapies often uses H&E to assess wound viability or depth of

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injury; however, H&E alone is insufficient to identify viability, as demonstrated by Sherwood and Flotte using lactate dehydrogenase (LDH).[9] LDH, a cytoplasmic enzyme found in nearly all living cells, catalyzes the conversion of pyruvate and nicotinamide adenine dinucleotide-H (NADH) to lactic acid and NAD^+. The LDH assay detects the presence of LDH enzyme in living cells by converting soluble nitroblue tetrazolium to an insoluble blue-purple precipitate known as formazan salt. Formazan is primarily localized in the cellular cytoplasm of histologic sections.[10] The LDH enzyme is stable for 36 h in cells; therefore, this assay provides a reliable estimate of viability in tissues requiring processing after removal.[11] Given the discrepancy between H&E and LDH, it is critical to understand the limitations of H&E as a method of detecting cell viability in burn injured tissues. We propose a simplified method of an LDH assay as a more accurate method of assessing cell viability *in situ* in burn tissue.

Materials and methods

Tissue collection and processing

To study cell viability in human burn injured tissues, informed consent was obtained to collect human burn tissue excised during the surgical procedure before skin grafting. This study was approved by the University of Wisconsin Human Subjects Committee Institutional Review Board in compliance with the 1975 Declaration of Helsinki. Intraoperatively, burn wound depth was determined using visual assessment by the burn surgeon. Six wounds clinically assessed as deep partial thickness (DPT) burns, and four wounds identified as FT burns were biopsied using a 4 mm sterile biopsy punch (Integra, York, PA). The area of wound surrounding the biopsy punch was then removed in a sequential manner by tangential excision to encompass the area of the biopsy. Tissues were maintained in the correct anatomic alignment (noting superficial and deep surfaces), and black indelible dye was placed on the most superficial side of the samples to maintain orientation. The burn injury of these samples occurred between 6 and 14 d before excision of tissue. Normal skin tissue was also collected from plastic surgery procedures as a normal control. Control and burn tissues were stored for up to 1 h in normal saline before further processing. After dividing the tissues in half, one half was fixed in 10% buffered formalin before paraffin embedding. Five-micron thick paraffin-embedded sections were stained with H&E. The other half of the tissues of each type was washed with phosphate buffered saline (PBS) and fixed in 1% paraformaldehyde for 2 h on a shaker at 4°C, followed by overnight incubation in a 20% sucrose solution at 4°C. The following day, the fixed and sucrose-dehydrated tissues were oriented in Tissue Tek OCT Compound (Fisher, Hampton, NH), and 10-micron thick cryostat sections were prepared.

In situ *LDH assay*

Previous LDH staining methods were modified and simplified for use with frozen sections.[9,11] Cryostat sections were dried at room temperature for at least 2 h but no more than 18 h. Sections were washed twice with PBS for 5 min each. Sections were incubated with freshly prepared LDH solution[11] containing 5% Polypep (Sigma, St. Louis, MO), 2-mM Gly-Gly (Sigma), 0.75% NaCl (Fisher), 60-mM lactic acid (Dot Scientific Inc, Burton, MI), 1.75 mg/mL ß-nicotinamide adenine (Sigma), and 30-mg Nitro blue Tetrazolium (Sigma) pH 8.0 for 3.5 h at 37°C. Slides were washed twice for 2 min each with 50°C tap water, followed by two washes of PBS of 2 min each. Tissues were counterstained with aqueous eosin (Newcomer Supply, Middleton, WI) for 4 min. Slides were washed with PBS for 1 s, dehydrated with acetone for 30 s followed by acetone: xylene (1:1) for 1 min, and finally with xylene alone for 1 min. Slides were then coverslipped with Permount (Vectors, Burlingame, CA). Digital images of H&E and LDH-stained slides at 10X and 40X were captured on a Nikon DS-Ri2 camera and Nikon Elements BR 4.51.00 software (Nikon, Tokyo, Japan).

Results

In our initial modifications of previously published protocols,[9,11] viable cells in the tissue could be clearly distinguished immediately following the staining procedure (data not shown). Final fixation of the tissue samples with 4% normal buffered formalin after eosin staining is a routine step in histologic procedures; however, with the initial modification of the LDH protocol, this step resulted in moisture retention and loss of tissue architecture as early as the day after staining, as illustrated by the arrows in Figure 1. After adapting the dehydration method using a series of alcohol and xylene washes to allow sufficient blue stain to remain as described in Sherwood and Flotte,[9] we found that this dehydration process resulted in a loss of our eosin stain (data not shown). This technical issue was ameliorated with a change from ethanol-based eosin to aqueous eosin, allowing simultaneous staining with eosin and LDH (Fig. 1). In Table 1, we present the procedural steps for *in situ* LDH staining in skin tissue and the modifications that were developed to overcome challenges associated with performing this technique on frozen sections of skin tissue.

Figure 2 illustrates the results using the final modified version of the *in situ* LDH assay on frozen sections of thermally injured human skin tissue. Staining patterns were uniform, reproducible, and consistent even after a month at room temperature after staining. Control samples showed that blue formazan granules were strongly localized in the cytoplasm of the epidermal keratinocytes in the normal tissue, as well as in the epithelial lining of the hair follicles, eccrine glands, and fibroblasts in the normal and the DPT burn tissue. A clear line of demarcation is evident in the LDH-stained DPT burn tissue that is not as obvious with H&E alone (Fig. 2 bottom panel). As would be expected, there is a lack of LDH staining in the first and most of the second tangential excision of the FT burns, consistent with the expected depth of injury. These results demonstrate that the modified LDH staining protocol to detect enzymatic activity was successful in clearly defining tissue viability in frozen sections of thermally damaged tissues *in situ*. The patients whose samples were included in this study all underwent successful autografting of their burns after