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Quantitation of Epidermal and Mucosal Tissue Injury Using Contrasts Agents and Imaging Techniques

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Abstract

Background/Purpose—Epidermal injury is common but the accuracy of visual methods is significantly impacted by the inherent skin pigmentation. We examined imaging and fluorescence techniques to quantify tissue injury as a function of skin color.

Methods—Epidermal and mucosal scratches were created in 20 light (L^* 68.2 ± 2.3) and 20 dark skinned (L^* 46.4 ± 5.2) females. Injured and uninjured sites were treated with toluidine blue (TB), fluorescein (FL) and a TB/FL mixture and photographed under conditions of white and fluorescent light. *Area and intensity* parameters were determined.

Results—Injured sites with TB and TB/FL had higher *areas* than the control for both light and dark subjects (ANOVA, $p < 0.05$). The *intensity* of the injured TB site was higher than the control for light skin only. The *areas* of injured sites with FL and TB/FL were higher than the control for both groups as were the *intensities* of the injured sites with FL. The findings were similar for the lip skin.

Conclusions—Application of TB and FL contrasts under white and fluorescent light can be used to quantify tissue injuries for L^* values > 35 and is a promising approach for the quantitation across a range of skin pigmentation.

Keywords

skin imaging; epidermal injury; mucosal injury; skin barrier compromise; digital image analysis; fluorescence imaging; skin pigmentation; quantitation of skin damage

INTRODUCTION

Epidermal skin injury can be caused by mechanical trauma (friction), exposure to solvents and chemical irritants, heat (burns) and environmental conditions (e.g., high or low humidity). Stratum corneum (SC) barrier compromise allows penetration of irritants into the dermis. Dryness (low moisture), scaling, fissuring, erythema (inflammation) and itching can occur as cellular repair mechanisms are upregulated. The selection and design of treatments

depends upon the availability of objective, quantitative methods for measuring the type and extent of injury in vivo.

Minor abrasions, scratches and fissures are characterized by erythema (redness), edema, exudate, and minor bleeding, usually surrounded by areas of normal, non-damaged skin. The extent of injury is typically determined with visual scoring methods based on severity (e.g., slight, mild, moderate, severe) and area (percent) of tissue damage. However, the accuracy of visual methods is significantly impacted by the inherent skin pigmentation, varying from very light (Caucasian) to very dark (African). Skin color results from constitutive pigments, e.g., melanins (yellow to brown), carotene (yellow), oxygenated hemoglobin (red), and reduced hemoglobin (purple-blue) and is determined by diffuse reflection and scattering and absorption of light inside the skin(1). Skin color differences are related to the melanin and the other chromophores. Erythema is difficult to assess in the darker skin due to masking by the red, yellow and brown pigments and tissue damage may be underdetermined.

Contrast dyes, such as toluidine blue, have been used to highlight tissue damage. Toluidine blue adheres to abraded or fissured nucleated cells in the epidermis, but does not bind to the normal anuclear cells(2). It can differentiate damaged from normal tissue and quantifying injuries in light skin but provides less contrast in dark-skinned subjects. The interference of pigmentation and limitations of visible spectrum contrast agents may be overcome by using fluorescent dyes. The light emitted at specific wavelengths is outside the regions of skin erythema and pigmentation (melanin). Fluorescein, a specific agent, has been used effectively to disclose tissue abrasion in the eye(3) for sentinel node mapping in tumors(4), for air leakage in pneumothoraces(5), cerebrospinal fluid fistulas(6), and for plaque and gum disease(7). Fluorescein was mixed into facial peel formulations (trichloroacetic or salicylic acid) and used with a Woods Lamp to insure uniform application of the agent in vivo(8). Intravenous fluorescein administration combined with a skin scratch test predicted the viability of skin flaps in dark-skinned patients(9). Fluorescein excitation in the visible range and photography were successful for evaluation of skin flap procedures(10). Fluorescence is low in normal skin where the surface pH is 5 – 5.5. The pH of injured tissue is higher, 6 – 7, due to exposure of the epidermis and fluorescein emits a strong green light under blue illumination in this range. We examined the use of digital imaging techniques, specifically, fluorescence imaging, as a method to detect and quantify the extent of epidermal and oral mucosal skin injury as a function of skin pigmentation. Normal and injured skin was treated with contrast agents toluidine blue, fluorescein, and mixtures and photographed under appropriate lighting (white light, blue light, respectively). Image analysis methods were used to determine both area and intensity of the normal and injured regions.

Materials and Methods

Subjects

Forty (40) healthy females, aged 18 or older, participated in January – February of 2007. Subjects had either (1) light skin of Type I/II (Fitzpatrick Scale) and a measured skin lightness (L^*) of 63 - 72 or (2) dark skin of Type V/VI and L^* of 35 - 53. Subjects were free of visible skin damage or irritation (e.g., dryness, irritation, dermatitis) and conditions that would influence the measurements (e.g., tattoos, scarring, moles) at the test sites had no allergies to nickel, fluorescein, lidocaine, epinephrine, food, animals, wool, cosmetics or skin care products. The Institutional Review Board of Cincinnati Children's Hospital Medical Center approved the research protocol. All subjects provided written informed consent and did not use topical skin lotions for 24 hours prior to the procedures..

Measurements

Quantitative Skin Color—The inherent skin color (unexposed area, inner aspect, upper arm) was derived from values of L^* (lightness), a^* (red/green color), and b^* (blue/yellow color) measured with a reflectance spectrophotometer (Color Tec-PSM® hand-held spectrophotometer). The L^* , b^* , and melanin index values correlate with the amount of epidermal melanin and skin pigmentation²⁴.

High Resolution Digital Imaging—The digital imaging system consisted of two Nikon D80 cameras with 10.2 Megapixels SLR, a 2 GB Micro Hard Drive, a Canfield TwinFlash, Canfield Polarizing Filters holder, and an AF Micor-Nikkor 60 mm f/2.8 Macro lens. Images were taken under controlled lighting conditions and standardized for white balance and color via standards within the image field. They were recorded with a speed of 125, an aperture of 32 and a lens distance of 61 cm from the surface of the imaging table. The digital images (JPEG format) were 2848×4256 with a resolution of 72 pixels/inch (2/8 pixels/mm). Each forearm was positioned on a vertical metal arm affixed to a black imaging platform (to avoid light reflection during image capture) at the specified distance from the lens. Color images were captured in the RGB format (Figure 1). Fluorescence images were taken of the same areas with a blue filter (Kodak #47A) on the ring flash and a yellow filter (Kodak #12 Deep Yellow Wratten Gel). Each subject positioned her lower lip for image capture.

Image Processing—The *extent of injury* was determined from the *area* and *intensity* of the injury using image processing methods. JPEG images were converted to the TIFF format (to prevent loss of detail, Adobe® Photoshop®), corrected using images of color standards (photographed during the same session) and subdivided into treatment areas. Following red channel extraction, toluidine blue areas (darker regions) were identified with ImageJ (.public domain, java based imaging software, National Institutes of Health) The background intensity (mean, σ) of the uninjured (control) was determined. For contrast treated sites, the region of interest threshold was set at -2σ and the mean intensity, σ and number of foreground pixels was recorded. The green channels were extracted from the fluorescence images. The mean and σ for the region adjacent to the treated area were computed to determine background fluorescence on fluorescein treated and uninjured skin. The area was selected, the threshold set at $+2\sigma$ and the mean intensity, σ and number of foreground pixels were recorded (Figure 1C and D). Similarly, mean and σ were measured and computed for the uninjured control sites.

Experimental Procedures

Two in vivo clinical model systems were used, i.e., the volar forearm as a model for epidermal injury and the inner lower lip for mucosal tissue injury. Use of both volar forearms allowed for six within subject sites for multiple treatment comparison and evaluation of the three contrast agents: toluidine blue (TB), (b) fluorescein (FL), and (c) a toluidine/fluorescein mixture (equal proportions) (TB/FL), each on injured (scratched) skin and an uninjured control site. To create the skin injury, two sets of superficial skin scratches were made with a sterile, latex free skin puncture device (Multi-Test® II, Lincoln Diagnostics, Inc.) on two of the six $2.5 \text{ cm} \times 5 \text{ cm}$ skin sites (4 scratched sites). The procedure created breaks in the skin, exposed epidermal fluid, causing erythema and edema. The treatments were applied using a pseudo-randomized complete block design to insure equal exposure and account for site variability: (1a) Scratched site #1 without any contrast, (1b) scratched site #1 with 1% FL (2a) scratched site #2 with TB, (2b) scratched site #2 with TB/FL mixture, (3) unscratched with 1% FL, (4) unscratched with TB, (5) unscratched with a mixture of a TB/FL mixture, and (6) no treatment (unscratched, no contrast). Excess contrast was blotted with a sterile gauze pad after one minute Color and fluorescence images

were recorded. The lower lip injury consisted of small lacerations made by a dentist (Dock) using the puncture device. The subject could elect have a local topical anesthetic (lidocaine gel) prior to the injury procedure. Color and fluorescence images were taken of the untreated scratched tissue. FL was applied to the scratched site and an adjacent uninjured control site. Excess contrast was blotted and images were taken.

Statistical Analysis

JMP Discovery Software (Statistical Analysis Systems) was used for the statistical procedures. The light and dark skinned groups were compared for age and lightness (L) using the Student's t-test (significance at $p < 0.05$). The outcome measures were evaluated separately for light and dark skinned groups. The effect of each contrast agent was determined by comparing the area and intensity relative to contrast treated uninjured sites as follows: (1) TB injured, TB uninjured, TB/FL injured and TB/FL uninjured were compared (one-way Analysis of Variance, significance at $p < 0.05$). (2) Similarly, FL injured, FL uninjured, TB/FL injured and TB/FL uninjured were compared (one-way ANOVA). Dunnett's Method was used for pairwise comparisons to the TB/FL uninjured control. The images of the FL treated injured mucosal sites were compared to the uninjured control for area and intensity using one-way ANOVA and Dunnett's Method.

Results

Examples of injured and uninjured skin sites treated with FL and the TB/FL mixture and taken under white light and fluorescence conditions are shown in Figures 1A and 1B, respectively.

Subjects

The mean skin lightness (L) for the light skinned group ($n = 20$) was 68.2 ± 2.3 (mean $\pm$ SD) with a range of 63.0 – 72.2. The average lightness (L) for the dark skinned group ($n = 20$) was 46.4 ± 5.2 with a range of 34.9 – 47.6. The L values were significantly different (student's t-test, $p < 0.05$). The mean age was 36.1 ± 8.2 years (range 23 – 58) for the light skinned group and 34.9 ± 10.7 years (range 24– 51) for the dark skinned participants. The groups were not significantly different in age.

Epidermal Tissue Injury: Toluidine Blue

The mean *area* and *intensity* results for injured TB, injured TB/FL, uninjured TB, and uninjured TB/FL for the light and dark skinned groups are shown in Figure 2. The injured sites treated with TB had a significantly higher *area* than the uninjured TB/FL control for both groups (Figure 2A). The uninjured TB/FL site served as the control (ANOVA, $p < 0.05$). The areas of the injured sites treated with TB and TB/FL were not significantly different and both were significantly higher than the control. The *intensity* of the injured site with TB was significantly higher than the control for the light skinned group only ($p < 0.05$, Figure 2B). The intensities of the injured sites with TB and with TB/FL were not significantly different for either group. The intensity of the injured site with TB/FL was not significantly different from the uninjured control for either group.

Epidermal Tissue Injury: Fluorescein

The mean *area* and *intensity* results for injured FL, injured TB/FL, uninjured FL, and uninjured TB/FL for the light and dark skinned groups are shown in Figure 3. The injured sites treated with FL and with TB/FL had significantly higher *areas* than the uninjured TB/FL control for the light and dark skinned groups (Figure 3A). The areas of the injured sites with FL and TB/FL were not significantly different for either group. The injured sites with

FL had a significantly higher *intensity* than the uninjured TB/FL control for both groups (Figure 3B). The intensities of the injured sites with FL and TB/FL were not significantly different for either group. The injured site with TB/FL had a significantly higher intensity than the uninjured TB/FL control for the light skinned group only (Figure 3B).

Mucosal Tissue Injury: Fluorescein

The mean area and intensity of the injured and uninjured lip tissue sites for FL contrast are shown in Table 1. The area was significantly greater for the injured site than the uninjured control for both groups. The intensity was significantly greater for the injured site than the uninjured control for the light skinned group and directionally higher ($p = 0.06$) for the dark skinned subjects.

Discussion

Visual skin methods commonly use descriptors such as slight, mild, moderate, severe to indicate severity of damage, e.g., inflammation and erythema(11,12). An advantage is that they are clinically relevant and rely on experience with deviations from the “normal” skin features. A disadvantage is that the methods have no “standards” for normal skin integrity and color making them difficult to implement uniformly across institutions and geographies. The observed skin color arises from the interplay of light with components of the stratum corneum, epidermis and dermis(13). Inherent skin pigmentation (i.e., brown, yellow, red colors due to melanin) influences the interpretation of visual erythema. Ben-Gashir reported that skin pigmentation, particularly in dark skinned patients, influenced the assessment of erythema in atopic dermatitis and cautioned that the severity could be underestimated(14). The assessment of the nonblanchable erythema of stage I pressure ulcers is a recognized limitation of the current staging system(15). Changes in blood, e.g., pooling in the tissues, affects the appearance of skin pigmentation(13). Photographic and imaging techniques have been developed to improve the objectivity and quantify the particular skin features (e.g., erythema, depigmentation, abrasion)(16-18). Under standardized conditions (lighting, focal length, white balance and color correction) photographs serve as a permanent clinical records and can be evaluated by multiple examiners.

Fluorescein is commonly used to identify tissue abrasion in the eye(3) and to assess epithelial wound healing rates with keratectomy procedures(19). Intravenous delivery of fluorescein has been used to enhance hypoperfusion in an animal model for evaluating injury to tissues of the trachea and larynx after intubation (20). Fluorescein derivatives (e.g., fluorescein isothiocyanate) have been attached to specific molecules to monitor specific structures, sites of reaction, tissue locations, etc., typically using in vivo fluorescence microscopy(21-23). Digital photography with blue light for excitation and a fluorescence filter was used to determine the tear meniscus height in patients with dry eye(24). Photography and image analysis were used to study the interaction of fluorescein with intraocular lens materials(25). The size and arrangement of stratum corneum corneocytes were evaluated with fluorescein contrast and photographic image capture (optical fiber bundle) at local sites(26). The present study is the first to use fluorescein contrast, digital imaging and fluorescence excitation to quantify epidermal and mucosal tissue injury in vivo.

Contrast agents (toluidine blue, fluorescein), high resolution digital photography and image analysis (both color and fluorescence modes) can be used to quantify epidermal and mucosal tissue injuries in vivo. Tissue damage is quantified from area of involvement and intensity, with a higher intensity indicating a greater concentration of the contrast agent. Injured skin was differentiated from the uninjured controls in both light and dark skinned subjects. The injured sites had significantly greater *areas of involvement* than the controls in both groups for all three contrasts. Fluorescein produced significant differences in *intensity* for injured

versus uninjured sites in both groups. while toluidine blue differentiated the sites in the light skinned group only. No differences in intensity were found for the toluidine/fluorescein mixture. Use of fluorescein contrast and high resolution digital photography with fluorescence excitation provides significant differentiation of minor epidermal and mucosal tissue injury across a range of light and dark pigmentation, i.e., lightness (L) values ≥ 35 (darkest pigmentation of the study population)..

Implications

The outcomes suggest that the tissue contrast and imaging methods can be used to quantify the severity of tissue injury and applied to other situations of epidermal and/or stratum corneum trauma, including abrasions, skin stripping, fissuring and inflammatory conditions (e.g., atopic dermatitis) where skin pigmentation interferes with visual methods. The method provides detection of tissue injury without interference from inherent skin pigmentation and, therefore, is a promising technical approach to clinical assessment of skin condition. Further investigations are warranted including evaluation on deeply pigmented skin, i.e., L values < 35 , and optimization of contrast concentration (to insure robustness of the system).

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Figures 1.

A and 1B. Sites (a) and (c) were injured and treated with FL and TB/TL, respectively. Sites (b) and (d) were uninjured and treated with FL and TB/FL, respectively. Figure 1A is the normal color image and 1B is the same area photographed under fluorescence conditions.

Figures 1C and 1D. The green channel has been extracted from the fluorescence image. Background region, (a), is selected and the mean and standard deviation (σ) is determined. The injured area is selected, as indicated by (c). Within region (c), a smaller area serves as the uninjured control site, indicated by (b). The mean and σ of the background fluorescence of FL uninjured tissue (b) are computed. The numerical value of the mean + 2σ of region (b) is set as the threshold for the injured area of region (c). The area and intensity are then computed.

Figure 2.

A. The injured sites with TB and TB/FL had significantly higher *areas* than the uninjured TB/FL control for both groups ($p < 0.05$). The areas of the injured sites with TB and TB/FL were not significantly different. The area of the uninjured TB site was not different from the uninjured TB/FL control. **Figure 2B.** The *intensity* of the injured site with TB was significantly higher than the TB/FL uninjured control for the light skin group only. The intensities of the injured sites with TB and TB/FL were not significantly different. The intensity of the injured site with TB/FL was not significantly different from the uninjured control for either group. *Indicates a significant difference ($p < 0.05$) versus the uninjured TB/FL control site.

Figure 3.

A. The injured skin sites with FL and TB/FL had significantly higher *areas* than the uninjured TB/FL control for both groups ($p < 0.05$). The areas of the injured sites with FL and TB/FL were not significantly different for either group. **Figure 3B** The injured sites with FL had a significantly higher *intensity* than the uninjured control with the TB/FL for both groups ($p < 0.05$). The intensities of the injured sites with FL and TB/FL were not significantly different for either group. The injured site with TB/FL had a significantly higher intensity than the uninjured TB/FL control for the light skin group only. *Indicates a significant difference ($p < 0.05$) versus the uninjured TB/FL control site.

Mean area and intensity values for the injured and uninjured lip sites for the fluorescein contrast with *fluorescence lighting* are shown. The uninjured site was the control for the statistical analysis. Values are reported as mean $\pm$ standard error.

Table 1

Caucasian					
Treatment (n)	Contrast	Area	P value**	Intensity	P value**
Injured (19)	Fluorescein	14.2 $\pm$ 2.29	0.0004	111.0 $\pm$ 5.7	0.01
Uninjured (19)	Fluorescein	1.55 $\pm$ 2.29	----	89.8 $\pm$ 5.7	----

African American					
Treatment (n)	Contrast	Area	P value**	Intensity	P value**
Injured (18)	Fluorescein	11.9 $\pm$ 1.32	<0.0001	76.0 $\pm$ 6.0	0.06
Uninjured (18)	Fluorescein	1.23 $\pm$ 1.32	----	59.4 $\pm$ 6.0	----

** P value versus control