Plasma Free Hemoglobin: A Novel Diagnostic Test for Assessment of the Depth of Burn Injury

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Background: Accurate determination of the depth of burn injury is difficult, even for experienced surgeons. The authors hypothesized that the level of plasma free hemoglobin following burn injury is correlated to the depth of burn injury, and they evaluated this hypothesis in a murine model.
Methods: Full-thickness and partial-thickness burn injuries of varying sizes were inflicted on 38 and 36 male Wistar rats, respectively. Blood specimens were taken at 0, 15, 30, 45, and 60 minutes after burn injury, and the levels of plasma free hemoglobin were determined spectrophotometrically.
Results: Full-thickness burns cause two times more hemolysis than partial-thickness burns ($p < 0.05$). A linear correlation was demonstrated between plasma free hemoglobin levels and total body surface area burned in both the full-thickness ($r = 0.91$, $p < 0.001$) and partial-thickness burn groups ($r = 0.94$, $p < 0.001$). The correlation between the quantity of hemolysis and the total body surface area burned was strongest at 15 minutes after the onset of burn injury. The levels of free hemoglobin peaked rapidly between 15 and 30 minutes after thermal injury and declined thereafter.
Conclusions: The authors' data suggest that the level of plasma free hemoglobin after burn injury is related to the size and depth of burn injury. This test can potentially be a valuable diagnostic adjunct in the assessment of burns. (*Plast. Reconstr. Surg.* 117: 1206, 2006.)

Burn size and depth, the presence of inhalation injury, and patient comorbidities are major prognostic factors shown to affect survival after burn injury.[1] Accurate early determination of burn size and depth is of paramount importance, as these factors have a direct bearing on the acute management of burn injuries. Burn size determines the need for immediate commencement of fluid resuscitation, and burn depth determines the need for early tangential excision.[1] The estimation of burn size and depth is a subjective clinical assessment requiring clinical acumen and experience.[3–8] There are currently no biochemical or hematologic tests that can detect the presence of deep dermal or full-thickness burn injuries. An objective laboratory test for the assessment of depth of burn injury that is routinely available would be a very helpful diagnostic adjunct in the assessment of burn injuries. The potential marker we evaluated in this study is the plasma free hemoglobin. We aimed to correlate the depth of burn and total body surface area burned with the plasma free hemoglobin level and to evaluate its use in the assessment of burn injuries.

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MATERIALS AND METHODS

Animals

Male Wistar albino rats were obtained commercially (Laboratory Animal Center, Sembawang, Singapore). The protocol for this study was approved by the Institutional Animal Care and Use Committee of the Singapore General Hospital before commencement. Rats weighing 571 g (SD, 68 g) were used for these experiments. They were kept at the Animal Holding

Facility at the Department of Experimental Surgery, Singapore Gen-eral Hospital, under standard conditions at 23 ± 1°C and were given rodent chow and water ad libitum. The guidelines for laboratory animal care were followed throughout the experiments.

Thermal Injury

The animals were anesthetized with intramuscular ketamine hydrochloride (75 mg/kg) and diazepam (5 mg/kg) before commencement of the experiments while spontaneously breathing room air and were kept adequately anesthetized throughout the procedure until killed. Partial-thickness and full-thickness burn injuries were inflicted using previously described methods.[14,19] A partial-thickness (second-degree) burn was inflicted by exposure of the shaved lumbosacral skin to 70°C water for 30 seconds[14] and a full-thickness (third-degree) skin burn was inflicted by exposure of the shaved lumbosacral skin to 100°C water for 10 seconds.[19] The surface of the skin was promptly dried by rolling the animal on a towel after exposure to hot water. Histologic examination of the burn area confirmed that partial-thickness and full-thickness burns were achieved.[20] The histologic criteria used for the assessment of depth of burn injury included the extent of interstitial and cellular destruction and the precise level of blocked and patent vessels within the skin and subcutaneous tissue.[20]

Experiments

This was a prospective, randomized, controlled laboratory study. The animals were randomly assigned into partial-thickness and full-thickness burn groups. Under anesthesia, the rat's tail was amputated and baseline blood was collected. In each group, burn areas of varying sizes (0 to 40 percent of total body surface area) were inflicted. The burn injury was inflicted as previously described. The surface area of burn inflicted was calculated using a planimeter. Rubner's formula ($A = 9.1\ W^{2/3}$, where A = surface area in centimeters squared and W = body weight in grams)[21] was used to calculate the total surface area of the animal. The right internal carotid artery was then cannulated with a large-bore canula (20 gauge) to allow gentle aspiration of blood specimens in heparinized syringes without causing iatrogenic hemolysis. Blood specimens were taken at 15, 30, 45, and 60 minutes after the onset of burn injury. The rats were killed at the end of the experiments by intravenous injection of concentrated phenobarbitone (0.1 ml/kg). Skin specimens were examined histologically to confirm that full-thickness or partial-thickness burns were achieved.

Measurement of Hemolysis

The blood specimens were centrifuged at 3000 rpm for 15 minutes. Rat plasma was subjected to spectrophotometric analysis (Smart Spec 3000; Bio-Rad, Hercules, Calif.). Plasma was then diluted in phosphate-buffered saline (pH 7.4). A dilution of 1:10, 1:30, or 1:60 was used, depending on the amount of hemolysis, to allow for direct absorbance studies spectrophotometrically. The Soret band 414 nm was used for quantitative determination of hemolysis after the appropriate dilution of the plasma.[14,15]

Statistical Analysis

Statistical analyses were performed using SPSS statistical software (version 11.0; SPSS, Inc., Chicago, Ill.). A simple linear regression approach was used to test for a linear relationship between the plasma free hemoglobin levels and total body surface area burned in each group (i.e., full-thickness and partial-thickness burn groups). The strength of linear correlation was expressed as the Pearson correlation coefficient (r) with p value. A value of $p < 0.05$ was considered statistically significant. The difference between two linear regression lines was estimated by 95 percent confidence interval analysis for clinical studies software. The calculation was based on the following information using 95 percent confidence interval analysis for clinical studies software: numbers of rats with full- and partial-thickness burns (n), slopes of the regression lines, standard deviations of full-thickness and partial-thickness groups, and residual standard deviations of free hemoglobin levels at 15 minutes (Y – dependent variable).

RESULTS

Nature of Products in Plasma

Plasma obtained after thermal injury consistently demonstrated the characteristic Soret spectrum with three peaks (at 414, 541, and 576 nm), confirming the presence of free hemoglobin[22] (Fig. 1). The absence of absorbance at 624 nm excludes methemalbumin. When plasma was treated with sodium nitrite, the absorbance peaks at 541 and 576 nm were lost, coincident with a new peak at 630 nm. This correlated with the conversion of oxyhemoglobin to methemoglobin.[22]

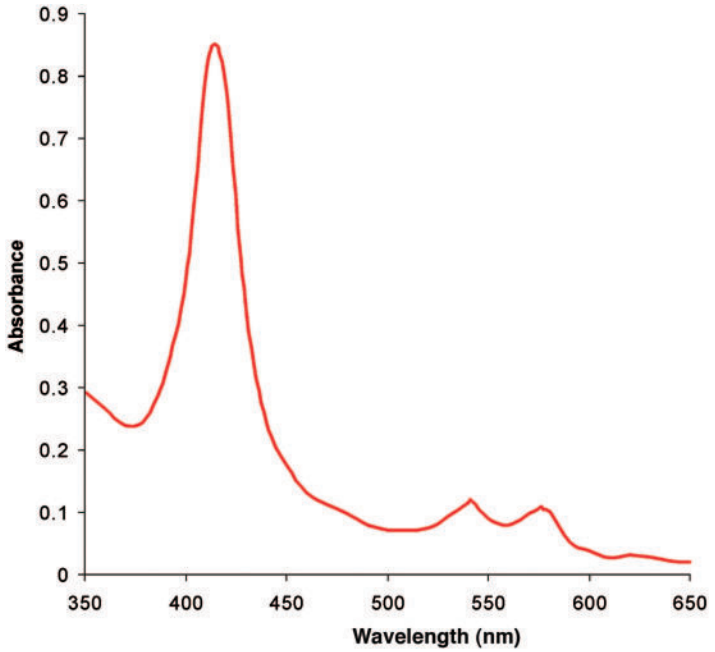

Fig. 1. Spectral analysis of serum after thermal injury demonstrated characteristic Soret bands of hemoglobin (at 414, 541, and 576 nm). The serum was obtained from rats 15 minutes after the onset of burn injury. This is consistent with the presence of free hemoglobin in the plasma.

These findings confirmed the presence of free hemoglobin in the plasma.

Forty male rats were randomly assigned into each burn group. Two and four rats in the full-thickness and partial-thickness burn groups, respectively, died before completion of the experiments and were not included in the analysis. Therefore, a total of 38 and 36 rats were studied in the full-thickness and partial-thickness burn groups, respectively. Rats subjected to thermal injury consistently demonstrated evidence of intravascular hemolysis. The time course and quantity of hemolysis in partial-thickness and full-thickness burns were, however, different in the two experimental groups (Fig. 2). For the full-thickness burn group, the maximal hemolysis was noted at 30 minutes following burn and remained elevated in the first hour after injury. In contrast, the amount of free hemoglobin in the plasma for the partial-thickness burn group peaked at 15 minutes postburn and declined rapidly thereafter (Fig. 2). At all times measured after the onset of burn injury, the amount of hemolysis in full-thickness burns was significantly higher than that seen in partial-thickness burn injury (Table 1).

Linear correlations were demonstrated between the total body surface area burned and the quantity of hemolysis as measured spectrophotometrically at 15, 30, 45, and 60 minutes after burn injury in both the full-thickness and partial-thickness burn groups. Using the Pearson correlation coefficient as a measure of strength of the linear association, this correlation was noted to be strongest at 15 minutes after burn injury (Table 2). At 15 minutes after injury, r values for the partial-thickness burn and full-thickness burn groups were 0.94 ($p < 0.001$) and 0.91 ($p < 0.001$), respectively. The difference between the two linear regression lines was statistically significant at 4.54 (95 percent confidence interval, 4.38 to 4.71). Thus, at 15 minutes after burn injury, for any percentage burn area, there was significantly more hemolysis in the full-thickness burn group, with approximately twice the amount of free hemoglobin detected in the plasma when compared with the partial-thickness burn group ($p < 0.05$) (Fig. 3).

DISCUSSION

We have demonstrated that acute thermal injury of rat skin leads consistently to intravascular hemolysis. This finding was also previously reported by several other authors both in animal models and in humans.[12–18] Two determinants of the quantity of intravascular hemolysis in acute burn injury demonstrated in this model were

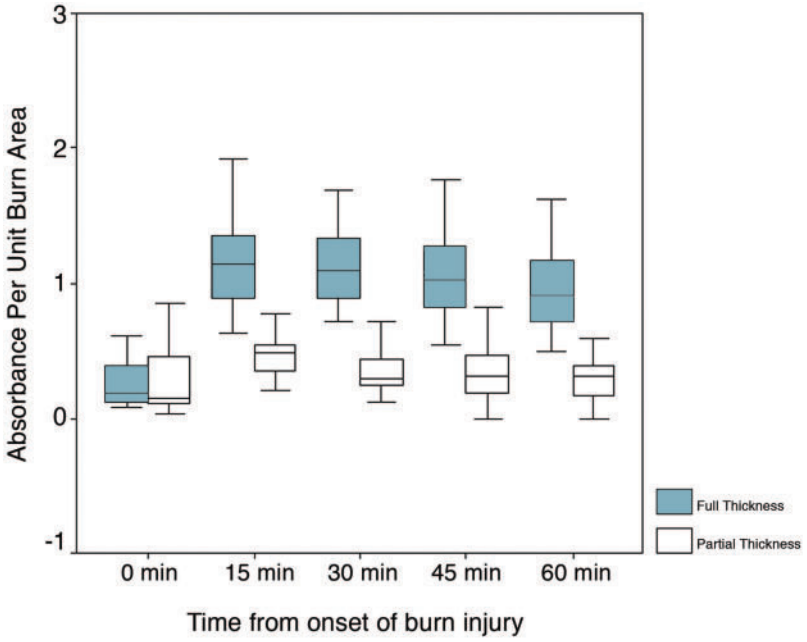

Fig. 2. Time course of hemolysis after the onset of full-thickness and partial-thickness burn injuries. *Boxes* represent the median and the interquartile ranges, and *error bars* represent the 5 to 95 percent range.

Table 1. Mean Absorbance of Plasma per Unit Burn Area for the Full- and Partial-Thickness Burn Groups at 0, 15, 30, 45, and 60 Minutes after Onset of Thermal Injury*

Time from Onset of Burn Injury	Mean Absorbance of Plasma per Unit Burn Area (absorbance/total body surface area)		*p* for Mean Difference between the Two Groups
	Full-Thickness Burn	Partial-Thickness Burn	
0 minutes	4.58	3.39	0.209
15 minutes	22.00	9.15	<0.001
30 minutes	22.05	6.43	<0.001
45 minutes	20.23	6.93	<0.001
60 minutes	18.68	6.33	<0.001

*At all times measured after the onset of burn injury, the amount of hemolysis in full-thickness burns was significantly higher than that seen in partial-thickness burn injuries.

burn depth and size. A linear correlation between burn size and the amount of intravascular hemolysis was demonstrated. Burn depth is the second determinant of the quantity of hemolysis, with full-thickness burn producing approximately double the amount of intravascular hemolysis as partial-thickness injury for a burn size of equal dimensions at 15 minutes after burn injury. Findings in this study suggested that measurement of intravascular hemolysis may potentially be a valuable diagnostic adjunct in the evaluation of depth burn injury.

Table 2. Pearson Correlation Coefficient for the Partial- and Full-Thickness Burn Groups at 15, 30, 45, and 60 Minutes after Onset of Burn Injury

	Time after the Onset of Burn Injury (minutes)			
	15	30	45	60
Pearson correlation coefficient, *r*				
Full-thickness burn group	0.91	0.92	0.84	0.83
Partial-thickness burn group	0.94	0.77	0.78	0.59

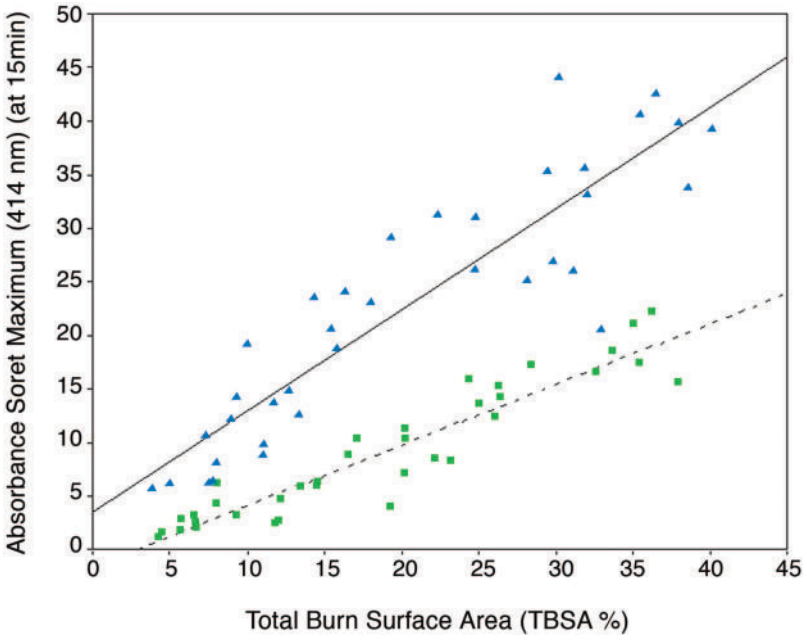

Fig. 3. Correlation between the amount of hemolysis (as measured by plasma absorbance at 414 nm) and the size of burn injury. A strong correlation was demonstrated in both the full-thickness ($r = 0.91$, $p < 0.001$) (*triangles*) and the partial-thickness ($r = 0.94$, $p < 0.001$) (*squares*) groups. The difference between the two linear regression lines was statistically significant at 4.54 (95 percent confidence interval, 4.38 to 4.71).

Loebl et al.[17] showed that red cells labeled with chromium-51 from burn patients when transfused into normal individuals have a normal lifespan. In contrast, they noted that red cells from healthy patients when transfused into burn patients have a markedly reduced survival. This suggested that the major mediator of hemolysis in burn injury was caused not by direct damage of the red cells by thermal trauma but by mediators in the plasma or vascular endothelium. Current evidence suggests that intravascular hemolysis is most likely mediated by systemic activation of the complement system. Complement activation results in the generation of C5a, which in turn activates neutrophils. The resultant respiratory burst and production of toxic oxygen products such as O_2^-, H_2O_2, and HO· is probably the direct cause of red cell destruction and intravascular hemolysis.[14,15,24] The linear relationship of the burned area and amount of intravascular hemolysis demonstrated in our study support the hypothesis that the thermally injured skin is critically involved in the process of complement activation.

Clinical assessment of burn depth is also frequently inaccurate.[6–8] The standard of care in burn patients involves early excision and grafting of all burns that will not heal within 3 weeks. Such burn wounds, when treated conservatively, often result in unsightly hypertrophic scars and functional impairment of the affected area. Also, in patients with deep dermal or full-thickness burns, early excision has been shown to reduce infective complications and sepsis and improve mortality.[25–28] Some authors even advocate acute burn excision within 24 hours of injury. The approach of serial evaluation has fallen out of favor, with many centers electing to excise clinically indeterminate burns.[29] The challenge in the acute management of burn injury today is therefore in the accurate identification of burns that will not heal within 3 weeks (i.e., deep dermal and full-thickness burns). Clinical assessment remains the standard for the diagnosis of burn depth. Very superficial wounds and very deep wounds present little difficulty to the experienced observer. However, there are many burn injuries whose burn depth is intermediate between the obvious ones. One study found that even evaluation by experienced surgeons of whether an apparently deep dermal burn will heal within 3 weeks has an accuracy of approximately 50 percent.[7]

Because of the limitations of clinical assessment and the proven benefit of early tangential excision of thermally injured skin,[25–28] numerous techniques and devices, such as laser Doppler flowmetry, thermography, ultrasound, and light reflectance, have been developed to determine burn depth more accurately than clinical observation alone.[6–10] Although these modalities are fairly accurate in the assessment of burn depth, use of these modalities has not gained widespread acceptance because they are expensive, time consuming, and uncomfortable for the patients to perform. Their use is currently largely restricted to the clinical trial setting.[8–10] Clinical evaluation therefore remains the standard of care. Plasma free hemoglobin obtained acutely after the burn injury can potentially serve as a diagnostic adjunct in the assessment of depth of burn injuries. Figure 4 shows our suggested scheme for evaluation of clinically indeterminate burn injury. Once the total burn surface area has been accurately determined, the level of plasma free hemoglobin can be used to infer the depth of the burn injury, with greater hemolysis being observed in deeper burns.

Some potential limitations to the use of free hemoglobin in the assessment of burns should be noted. Plasma free hemoglobin levels change rapidly with time from the onset of injury. It is rapidly released by red cell destruction at the onset of injury but also rapidly cleared from the circulation (by the kidneys and liver). The best time to obtain a blood sample for analysis is at 15 minutes after the onset of injury, as the correlation with the size of burn injury is strongest at this time (Table 1). After this time, the level tends to trend downward as hemoglobin is cleared from the circulation. This observation on the time course of intravascular hemolysis was also noted by other authors.[14,15] Furthermore, if acute complications of burn injury supervene (such as acute renal failure or hypotensive shock), progressive elevation may be observed. This explains the progressively increasing variability of the plasma free hemoglobin as time from the onset of injury increases observed in our study. Therefore, a blood specimen taken immediately after burn injury (i.e., at 15 minutes) would probably yield the most accurate results. In the clinical situation, this means that a blood specimen would have to be taken at the scene of the accident by paramedical staff, as it would take at least 2 hours for patients to be brought to the hospital.

The technique of blood sampling is also important. Gentle aspiration with a large-bore needle prevents iatrogenic traumatic hemolysis. The increased red cell osmotic fragility in thermal injury makes them susceptible to this complication, resulting in a spuriously high reading.[14,15,30] In the assessment of burn depth, it is essential to remember that burn injury is a dynamic process. Inade-

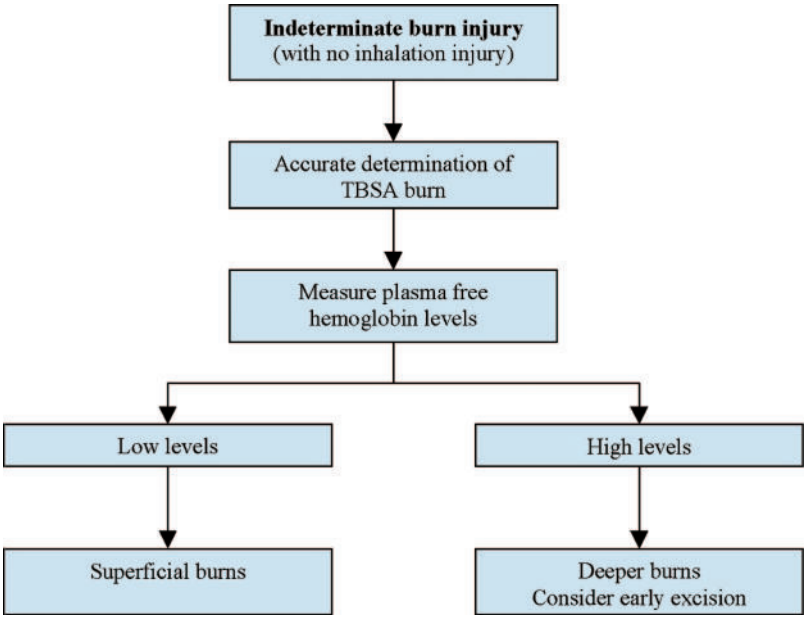

Fig. 4. Potential clinical application of plasma free hemoglobin levels for assessment of clinically indeterminate burns.

quate resuscitation can result in progression of skin loss, particularly in the zone of stasis.[31] Thus, a wound that is superficial at initial assessment (with consistent plasma free hemoglobin level) may convert to a deep dermal injury by day 3, necessitating excision. Free hemoglobin is a point measure reflecting the amount of thermal trauma at the time of injury. Serial clinical evaluation of the evolving burn injury therefore remains important.

CONCLUSIONS

This murine experimental model demonstrated the potential use of plasma free hemoglobin levels in the acute evaluation of burn injury. This is the first laboratory test correlated to the depth and size of acute burn injuries. Intravascular hemolysis is a surrogate marker of the severity of thermal injury and is affected by both the size and the depth of burn. Although there are limitations, when used in the right context, plasma free hemoglobin can potentially be a valuable diagnostic adjunct in the evaluation of thermal trauma. Caution should be exercised in extending these data to human subjects, as success in an animal model does not always translate into consistent findings in humans. This is certainly an area that deserves further research to validate and translate this marker into clinical use.

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