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Intraoperative Prediction of Post-Operative Flap Outcome using the Near-Infrared Fluorophore Methylene Blue

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Abstract

Methylene blue (MB) is a near-infrared (NIR) fluorophore that provides a stable visual map of skin perfusion after intravenous injection. We explored the capability of MB to predict submental flap postoperative outcome using a single intraoperative measurement. Submental flaps were created in N = 15 pigs and imaged using the FLARE™ imaging system immediately after surgery and at 72 h. Using the first 3 pigs, optimal MB dosing was found to be 2.0 mg/kg. Training and validation sets of 6 pigs each were then used for receiver operating characteristic (ROC) analysis. In the training set, a contrast-to-background ratio (CBR) threshold of 1.24 provided the highest sensitivity and specificity to predict tissue necrosis at 72 h. In the validation set, this threshold provided a prediction sensitivity of 95.3% and a specificity of 98.0%. We demonstrate that a single intraoperative NIR measurement can predict submental flap outcome at 72 h.

Keywords

Flap perfusion; predictive capability; methylene blue; near-infrared fluorescence imaging

INTRODUCTION

The prediction of flap viability during reconstructive surgery is still largely based on clinical judgment. Flap viability is important in both pedicled and free flaps because intraoperative revision can prevent long-term morbidity, such as flap loss, necrosis, and poor wound healing¹. Intraoperative assessment of perfusion is important because early and accurate detection of areas with insufficient perfusion increases the probability of successful revision. Most surgeons assess skin color, capillary refill, and dermal bleeding to predict viability; however, these subjective measures can often fall short. In addition, objective measures of flap perfusion such as thermometry², Laser Doppler Flowmetry³, pulse oximetry^{4,5}, and transcutaneous oxygen monitoring⁶ are rarely used. Among these methods, there is currently no accepted standard for evaluating flaps in the intraoperative setting^{7,8}.

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Recently published studies from our group^{9–13} and others^{14–16} suggest that invisible near-infrared (NIR) fluorescent light, used in conjunction with the blood pool agent and NIR fluorophore indocyanine green (ICG), provides real-time angiography of perforator vessels and can provide prediction of flap viability (reviewed in¹⁷). NIR fluorescence angiography using ICG has recently been optimized in a pilot clinical trial¹⁸.

Although ICG, which fluoresces at ≈ 800 nm, is the most widely used NIR fluorophore to date for assessing perforator vessels, the 700 nm NIR fluorophore methylene blue (MB) has certain advantages. Like ICG, it is already FDA-approved for other indications and thus available for eventual pilot clinical trials. Being a lipophilic, cationic small molecule (321 Da), MB is capable of being extracted by blood into living cells, thus acting as a perfusion tracer¹⁹. Unlike ICG, MB fluorescence is longer lasting in tissue and provides a convenient visual map of tissue perfusion.

The hypothesis guiding this study was that MB could provide quantitative assessment of flap perfusion and that a properly developed metric might permit prediction of long-term flap outcome based on a single intraoperative optical measurement.

MATERIALS AND METHODS

Near-Infrared Fluorophores

MB stock solution (1%, 10 mg/mL, 31.3 mM) was purchased from Taylor Pharmaceuticals (Buffalo Grove, IL). ICG powder was purchased from Akorn (Decatur, IL) and resuspended with 10-ml of supplied diluent to produce a 3.2 mM stock solution. All *in vitro* measurements were performed in 100% fetal bovine serum (FBS) supplemented with 50 mM HEPES buffer, pH 7.4. Absorbance and fluorescence were measured with fiberoptic HR2000 absorbance (200–1100 nm) and USB2000FL fluorescence (350–1000 nm) spectrometers (Ocean Optics, Dunedin, FL). A 5-mW, 654-nm laser diode provided NIR excitation. Fluorescence quantum yield (QY) of MB in FBS was calculated using oxazine 725 in ethylene glycol (QY = 19%²⁰) as a calibration standard under conditions of matched absorbance (absorbance = 0.08) at 654 nm. MB was diluted in 10-ml of saline to produce a final injected dose of 0.5, 1.0, or 2.0 mg/kg. MB was administered as a rapid intravenous bolus via the external jugular vein.

FLARETM Imaging System Components

The FLARETM imaging system has been described in detail previously by our laboratory^{21,22} and permits positioning of the custom optics anywhere in 3-dimensional space while maintaining a working distance of 18 inches between optics and the surgical field. Over a 15-cm field of view, 40,000 lx of white light (400–650 nm), 4.0 mW/cm² of 670-nm near-infrared (NIR) fluorescence excitation light, and 11.0 mW/cm² of 760 nm NIR fluorescence excitation light were generated by light emitting diodes (LEDs). Color video of the surgical field and 2 channels of NIR fluorescence images such as 700 nm NIR fluorescence and 800 nm NIR fluorescence were obtained simultaneously with 60 ms (16.7 Hz) or 500 ms (2 Hz) exposure times. After computer-controlled image acquisition via custom software, color video and NIR fluorescence images can be displayed individually and/or merged. The 700 nm and 800 nm NIR fluorescence channels were used for imaging MB and ICG, respectively.

Animal Usage

Animals were studied under the supervision of an approved institutional protocol. Fifteen female Yorkshire pigs averaging 34.8 kg were purchased from E.M. Parsons and Sons, Hadley, MA. Three pigs were used to determine the ideal dose of MB (non-survival study).

The remaining 12 pigs were divided into 2 groups: 6 pigs were used in a training set to determine the cutoff values between viable and necrotic areas, and 6 pigs were used as part of the validation set to prospectively confirm the previously determined cutoff values. Pigs were induced with 4.4-mg/kg intramuscular Telazol®, a combination of tiletamine and zolazepam purchased from Fort Dodge Animal Health (Fort Dodge, IA), intubated, and maintained with 2% isoflurane. Heart rate, ECG, oxygen saturation, and body temperature were monitored during the experiments. A 14G central venous catheter was inserted into the external jugular vein. After preparation, submental flaps (see below) were created. For survival experiments, antibiotics and analgesics were administered as follows: 5 mg/kg of Baytril® (enrofloxacin) purchased from Bayer Healthcare (Morristown, NJ) was injected before skin incision and postoperatively as an antibiotic, while 0.03 mg/kg of buprenorphine was injected 20 min before extubation. After injection of buprenorphine, a 2.5-µg/kg fentanyl patch was attached to the back of each pig. On the third postoperative day (POD#3), animals were anesthetized as described above using Telazol® and isoflurane. Flap perfusion was quantified again using MB.

Surgical Models

For identification of perforators, 0.070 mg/kg of ICG was injected intravenously as described previously^{10,13}. Once the perforators were identified, a 8 × 8 cm round submental flap was designed and created. Two or 3 perforators were preserved within the flap. For dose optimization, 0.5-, 1.0-, or 2.0-mg/kg MB was injected intravenously and imaging was performed continuously over 15 min using a 60 ms camera exposure time. For the training and validation sets, the optimized MB dose (2.0-mg/kg) was administered and a single NIR fluorescence image was taken at 5 min postinjection using a 500 ms camera exposure time. For each animal, MB imaging was performed intraoperatively (0 h) and on POD#3 (72 h).

Visual Evaluation

Prior to quantitative assessment, 3 independent experienced surgeons blinded to each other's results drew regions of interest (ROIs) corresponding to their personal determination of flap perfusion. For automated quantitation, ImageJ (National Institute of Health, Rockville Pike, Maryland, USA) was used.

Quantitative Assessment

At each time point, the fluorescence intensity (FI) of a ROI over the flap or background (BG) were quantified using custom software. Small circles were drawn as a ROI on all areas of the submental flap. To standardize the background measurement in each flap, we centered the background ROI (BG) over the silk suture that was used to secure the flap. The contrast-to-background ratio (CBR) was defined as $CBR = (FI \text{ of ROI} - FI \text{ of BG}) / (FI \text{ of BG})$. At 72 h, visible tissue necrosis (purple skin with no capillary refill) served as the criterion standard for poor perfusion.

Statistical Analyses

The optimum cutoff value for differentiation of poor perfusion (necrosis) from adequate perfusion was defined as the point on the receiver operating characteristic (ROC) curve with the minimum distance from the 100% true-positive and the 0% false-positive rate. The analyzed values were reported as mean ± SEM. All analyses were performed with GraphPad Prism software for Windows and Macintosh (Version 4.0, GraphPad Software Inc., La Jolla, CA). Sensitivity is defined as the percentage of ROIs that was determined as having poor perfusion by CBR cutoff, which were on POD #3 to be necrotic (true positive), divided by the total number of necrotic ROIs on POD #3 (Table 1). Specificity is defined as the percentage of ROIs that were determined as viable by CBR cutoff, which were found on

POD #3 to be viable (true negative), divided by the total number of viable ROIs on POD #3. Positive predictive value (PPV) is defined as the percentage of true positives divided by the number of ROIs that were determined to have poor perfusion by CBR cutoff. Negative predictive value (NPV) is defined as the percentage of true negatives divided by the number of ROIs that were determined to be viable by CBR cutoff. Finally, accuracy is defined as the percentage of true positive and true negative ROIs divided by the number of total ROIs.

RESULTS

Optical Properties of MB

In 100% serum, the peak absorbance and emission of MB were 665 nm and 688 nm, respectively. The extinction coefficient at peak absorbance was $71,200 \text{ M}^{-1}\text{cm}^{-1}$ and the QY was 3.8%.

MB Dose Optimization

The NIR fluorescence intensity of skin was found to be highly dependent on injected MB dose, with a non-linear relationship and a threshold effect (Figure 1). Both quantitatively (Figure 1A) and qualitatively (Figure 1B), a high dose of MB was required, with 2.0 mg/kg being chosen for survival studies because of its high signal and the fact that it is the upper limit of the dose FDA-approved for other clinical indications. MB fluorescence intensity peaked shortly after intravenous bolus injection, corresponding to peak arterial concentration, then was rapidly extracted by skin resulting in a relatively stable NIR fluorescence pattern throughout the skin by 5 min after injection. Skin NIR fluorescence then decayed slowly back to baseline over the course of ≈ 1 h.

Manual Quantitation of Perfused Area

Three independent observers, blinded to the imaging performed during surgery, were asked to draw the boundaries of those areas they considered to have low perfusion based on MB NIR fluorescence. As shown in Figure 2A, this task often resulted in high interobserver variability. A summary of results obtained using manual quantitation is provided in Figure 2B and demonstrates that observers consistently underestimated the area of perfusion that would be seen at POD #3 when using intraoperative perfusion maps, and that estimates made from intraoperative data had higher interobserver variability than those made at POD #3.

Automated Quantitation of Perfused Area and Creation of a Training Set for Predicting Long-Term Flap Perfusion

In $N = 6$ pigs comprising the training set, a series of small ROIs (65.3 ± 4.8 ROIs per flap) were drawn over the intraoperative NIR fluorescence image taken 5 min after MB injection (Figure 3A). Then CBR of all ROIs was calculated. The clinical viability of all ROIs was observed at 72 h. Intraoperative CBR and clinical viability at 72 h were compared to determine the CBR cutoff (i.e., threshold). Using receiver operator curve (ROC) analysis, it was found that a CBR cutoff of 1.24 was predictive of flap outcome seen at 72 h (POD #3). As shown in Figure 3B, a CBR cutoff of 1.24 resulted in an area under the ROC curve (AUC) of 0.9698 and a sensitivity, specificity, positive predictive value (PPV), negative predictive value (NPV), and accuracy for tissue necrosis of 91.8%, 92.0%, 67.5%, 98.4%, and 91.9%, respectively.

Validation of Training Set Results

In a second set of $N = 6$ pigs comprising the validation set, MB NIR fluorescence images were acquired intraoperatively ($T = 0$) and at 72 h postoperatively (POD #3), both 5 min

after MB injection. The same number of ROIs was drawn over the intraoperative image (Figure 4A) and computer software was used to flag any ROI falling below the CBR cutoff of 1.24. Results were then compared quantitatively to flap perfusion seen at T = 72 h (POD #3). As shown in Figure 4B, automated prediction of necrosis resulted in an AUC of 0.9969, and a sensitivity, specificity, PPV, NPV, and accuracy for necrosis of 95.3%, 98.0%, 96.0%, 97.6%, and 97.1% respectively.

DISCUSSION

Clinical examination based on color, capillary refill, and turgor can often be unreliable in assessing perfusion in both pedicled and free flaps. In addition, areas of necrosis and partial flap loss are often difficult to predict at the time of surgery and can manifest up to 3 days later. Objective assessment such as thermometry,² Laser Doppler Flowmetry,³ pulse oximetry,^{4,5} and transcutaneous oxygen monitoring⁶ cannot provide an accurate delineation between viable and non-viable areas intraoperatively.

Previously, Graham et al. used fluorescein to assess flap perfusion. Today, this technique is rarely used.²³ ICG is a second-generation dye that has largely replaced fluorescein, showing cutaneous blood flow in real time and with higher sensitivity due to lower background autofluorescence in the NIR.

Several studies have demonstrated the use of ICG angiography for assessing flap blood flow both preclinically and clinically. ICG fluorescence angiography has been used to identify perforators¹³, to evaluate microsurgical anastomoses²⁴, and to predict the viability of flaps¹⁶. Although some studies have used ICG to evaluate the viability of the flap using quantitative assessment^{25,26}, it is still difficult to determine the border between viable and necrotic skin. For example, although Giunta et al. showed an accurate cutoff value separating viable from necrotic area in rats using a NIR imaging system, the perfusion borders are not readily apparent²⁵. Also, in the early post-operative stage, the pattern of blood flow in a flap may not be the same as in the late post-operative stage. Our previous studies have shown that skin perfusion recovers gradually over 6 h, even if only one perforator is preserved¹⁰. Thus arterial spasm, A-V shunt flow, and capillary permeability, make it difficult to predict future perfused areas. Gordon et al. also reported that maximum blood flow occurred at 24 h after flap creation and maximal A-V shunt flow occurred at 12 h²⁷.

The phenothiazine derivative MB has chemical properties that distinguish it from ICG. Although its optical properties are not as good as ICG, that is, MB is about 3- to 5-fold less bright on a molar basis, its NIR fluorescence peaks at 700 nm. This permits use of MB with NIR Channel #1 of the FLARE[™] imaging system²² without interfering with NIR Channel #2 (used for 800 nm fluorophores like ICG). Second, MB is already FDA-approved for other indications and thus available for clinical investigations. Third, MB is also cleared into both bile and urine, permitting intraoperative mapping of bile ducts²⁸ and ureters²⁹, respectively. Fourth, unlike ICG, which binds to blood proteins and is first-pass extracted by the liver, MB is extracted by tissues such as skin during the arterial phase, resulting in relatively stable intracellular fluorescence¹⁹. It is the property of tissue extraction that makes MB a true perfusion tracer, as opposed to ICG, which is only a blood pool agent that transiently highlights arteries, capillaries, and veins. The stable MB NIR fluorescence of skin provides a visual map of perfusion using a single sub-second image, with relatively sharp borders between perfused and under-perfused areas (see, for example, Figure 2A).

Although not surprising, our study confirmed that manual quantitation of perfused area had high inter-observer variability. Using simple computer-assisted quantitation, however, a

CBR cutoff could be determined, which had high accuracy to predict flap perfusion on POD #3 using a single intraoperative image obtained immediately after flap creation. These results should be interpreted, though, within the limitations of our study. Our flap size (8 × 8 cm) was limited by the field-of-view of the imaging system. The results have not yet been confirmed on larger flaps. Our sample size was relatively small, and a slight improvement using the validation set relative to the training set suggests that a learning curve was present, which might affect the PPV. For example, we improved our ability to create reliable necrotic areas in the second group (validation set) of animals studied compared to the first group (training set), which resulted in a lower prevalence rate in the training set, reducing the PPV.

Although the MB optimal dose we found (2.0 mg/kg) is within the recommended intravenous clinical dose of 1–2 mg/kg³⁰, and is below the UK National Poisons Information Service maximum dose of 4 mg/kg of a 1% solution³¹, maximal NIR fluorescence was obtained using a rapid intravenous bolus. This method of administration is not approved for clinical use, and most clinical MB applications utilize slow infusion over 15 min to avoid high peak concentrations, which can lead to methemoglobin production. Serious adverse reactions to MB have been reported when used at doses of 7.5 mg/kg³¹. No adverse reactions occurred in any of the pigs administered 2.0 mg/kg, except for a transient and artificial lowering of SpO₂ due to MB NIR absorption, which interferes with the pulse oximetry instrument. Nevertheless, extrapolation of our results to initial clinical studies must be done with caution. Finally, the CBR threshold obtained in animal models is unlikely to be the same in humans, requiring a similar clinical study design that employs independent training and validation sets.

Despite these limitations, our study serves as the foundation for future pre-clinical and clinical investigations. Especially exciting is the possible combination of MB and ICG for flap assessment. MB fluorescence does not interfere with ICG fluorescence, and vice versa. The information provided by MB (perfusion) and ICG (vascular flow) are complementary. The FLARETM imaging system can image MB and ICG simultaneously, thus permitting the exploration of techniques that could further improve predictive value.

CONCLUSION

When administered as an intravenous bolus immediately after flap creation, MB produces NIR fluorescence that provides a visual map of skin perfusion. When combined with simple computerized analysis, a single NIR fluorescence image obtained intraoperatively can predict flap outcome on POD #3 with relatively high accuracy.

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A.

Dose Dependence of MB Signal Strength

B.

Figure 1. Methylene blue (MB) as a perfusion tracer and optimization of injected dose

A. Direct comparison of contrast-to-background ratio (CBR, mean $\pm$ SEM) over time for 0.5 mg/kg (white square), 1.0 mg/kg (white circle), and 2.0 mg/kg (black circle) of MB injected as a rapid intravenous bolus.

B. Actual near-infrared (NIR) images at peak fluorescence (≈ 25 s) obtained with 0.5 mg/kg (left), 1.0 mg/kg (middle), and 2.0 mg/kg of MB (right). Images have identical exposure times (60 ms) and normalizations. Scale bar = 3 cm.

Figure 2. Inter-observer variability in perfused area measurements using manual delineation

A. The 3 different colors represent the regions of interest (ROI) drawn by 3 independent observers when asked to define perfused area manually. Shown are intraoperative (0 h) and POD#3 (72 h) near-infrared (NIR) images taken 5 min after 2.0 mg/kg MB injection. Camera exposure time was 500 ms.

B. Perfused area (mean $\pm$ SEM) as measured in N = 6 pigs by 3 independent observers using images taken intraoperatively (0 h) and on POD#3 (72 h).

A.

B.

ROC Curve: Training

	Percentage (%)	95% CI
Sensitivity	91.8	83.6% to 96.3%
Specificity	92.0	90.5% to 92.8%
PPV	67.5	61.5% to 70.8%
NPV	98.4	96.8% to 99.3%
Accuracy	91.9	89.4% to 93.3%

Figure 3. Training set for perfused area measurements and its statistical analysis

A. Representative intraoperative (0 h) and POD#3 (72 h) images that were used for developing the training set for perfused area measurements. Shown are color video (left) and near-infrared (NIR) fluorescence images (right) taken 5 min after 2.0 mg/kg MB injection. Camera exposure time was 500 ms. Shown on the top right are the individual regions of interest (ROI) that were used for calculating contrast-to-background ratio (CBR). Scale bar = 3 cm.

B. Receiver operating characteristic (ROC) curves for prediction of perfusion on POD#3 from a single intraoperative CBR measurement. The point on the curve with the highest sensitivity and specificity represents the cutoff value (CBR threshold). AUC = area under the curve. 95% CI = 95% confidence interval. NPV = negative predictive value. PPV = positive predictive value.

A.

B.

ROC Curve: Validation

	Percentage (%)	95% CI
Sensitivity	95.3	92.0% to 97.2%
Specificity	98.0	96.3% to 99.0%
PPV	96.0	92.7% to 97.9%
NPV	97.6	95.9% to 98.6%
Accuracy	97.1	94.9% to 98.4%

Figure 4. Validation set for perfused area measurements and its statistical analysis

A. Representative intraoperative (0 h) and POD#3 (72 h) images that were used for the validation set for perfused area measurements. Shown are color video (left) and near-

infrared (NIR) fluorescence images (right) taken 5 min after 2.0 mg/kg MB injection. Camera exposure time was 500 ms. Shown on the top right are the individual regions of interest (ROI), with those shaded purple representing ROIs that were below the contrast-to-background ratio (CBR) threshold of 1.24, i.e., those that would theoretically predict poor perfusion at POD#3. Scale bar = 3 cm.

B. Receiver operating characteristic (ROC) curves for prediction of perfusion on POD#3 from a single intraoperative CBR measurement. The CBR threshold (cutoff) value used was 1.24. AUC = area under the curve. 95% CI = 95% confidence interval. PPV = positive predictive value. NPV = negative predictive value.

Table 1

2×2 Table Analysis

Measured CBR Value	Necrosis on POD #3	Viable on POD #3	
CBR Cutoff during surgery	True Positive (TP)	False Positive (FP)	PPV = TP/(TP+FP)
> CBR Cutoff during surgery	False Negative (FN)	True Negative (TN)	NPV = TN/(FN+TN)
	Sensitivity = TP/(TP+FN)	Specificity = TN/(FP+TN)	Accuracy = (TP+TN)/(TP+FP+FN+TN)

Abbreviations: CBR, contrast-to-background ratio; NPV, negative predictive value; POD, postoperative day; PPV, positive predictive value.