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Quantitative Measure of Intestinal Permeability Using Blue Food Coloring

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Abstract

Background: Loss of intestinal barrier integrity plays a fundamental role in the pathogenesis of various gastrointestinal diseases and is implicated in the onset of sepsis and multiple organ failure. An array of methods to assess different aspects of intestinal barrier function suffers from lack of sensitivity, prolonged periods of specimen collection, or high expense. We have developed a technique to measure the concentration of the food dye FD&C Blue #1 from blood and sought to assess its utility in measuring intestinal barrier function in humans.

Materials and methods: Four healthy volunteers and 10 critically ill subjects in the intensive care unit were recruited in accordance with an institutional review board approved protocol. Subjects were given 0.5 mg/kg Blue #1 enterally as an aqueous solution of diluted food coloring. Five blood specimens were drawn per subject: 0 h (before dose), 1, 2, 4, and 8 h. After plasma isolation, organic extracts were analyzed by high-performance liquid chromatography/mass spectrometry detecting the presence of unmodified dye.

Results: We found no baseline detectable absorption in healthy volunteers. After including the subjects in the intensive care unit, we compared dye absorption in the six subjects who met criteria for septic shock with the eight who did not. Septic patients demonstrated significantly greater absorption of Blue #1 after 2 h.

Conclusions: We have developed a novel, easy-to-use method to measure intestinal barrier integrity using a food grade dye detectable by mass spectrometry analysis of patient blood following oral administration.

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Keywords

Intestinal barrier; Gut permeability; Sepsis; FD&C Blue #1; High performance liquid chromatography/mass spectrometry; Critical illness

Introduction

The gastrointestinal tract is an extensive surface charged with a multitude of functions including the establishment of a selective barrier between the internal and external environments.[1] This consists of a physical barrier created by a mucus layer, a lining of enterocytes of epithelial origin, and various tight junctions, as well as an immunologic barrier maintained by immune-sensing cells and Paneth cells that release lysozymes and defensins. Mounting evidence implicates the disruption of intestinal barrier integrity in the development of numerous ailments such as inflammatory bowel disease and celiac disease.[2,3] Furthermore, loss of intestinal barrier function is frequently associated with the onset of sepsis and multiple organ failure, especially when combined with intestinal hypoperfusion in trauma or major surgery.[4,5] Abnormal blood flow, such as portal hypertension and cirrhosis, is also linked to reduced intestinal barrier function.[6,7] Disruption of the intestinal barrier facilitates translocation of toxins, bacteria, and other pathogenic entities into the circulation, thereby eliciting an inflammatory response that may result in sepsis and multiple organ failure.[8–12]

Over the past 20 years, an array of methods has been developed to assess different components of intestinal barrier function.[1,2] These include analyzing epithelial cell damage by quantitating fatty acid–binding protein[13–18] or glutathione S-transferase in blood or urine,[19–22] measuring paracellular barrier integrity loss by detecting claudin-3 in the urine,[23–26] and assessing functional barrier compromise using differential sugar absorption tests,[2,3,27–29] the detection of polyethylene glycols,[2,30–34] and chromium-labeled ethylenediaminetetraacetate in the urine.[35] The detection of plasma endotoxin[36,37] and D-lactate levels[38] have also been proposed as indirect methods of assessing intestinal barrier function. However, these tests are impractical due to prolonged incubation periods, lack of sensitivity and specificity, difficulty obtaining large amounts of sample, such as 24-h urine studies, and high cost.[1,2,39–41]

A recent study evaluating the intestinal permeability of fruit flies demonstrated that FD&C Blue No. 1 (Blue #1) can be used effectively for this purpose.[42] With Blue #1, the authors demonstrated an association between loss of intestinal integrity and altered metabolic and immune signaling. Importantly, their findings showed that loss of intestinal integrity was the harbinger of death. Animal studies indicated that Blue #1, which is a water-soluble triphenylmethane dye, is minimally absorbed from the gastrointestinal tract and has rapid biliary and renal excretion with low metabolic transformation.[43–46] Remarkably, long-term studies have revealed no associated genotoxicity, physiologic, reproductive, or carcinogenic effects.[47,48] Approved for use in foods, drugs, and cosmetics by the U.S. Food and Drug Administration since 1969, Blue #1 has a recommended maximum daily intake of 12 mg/kg body weight.[45,49,50] Based on observed safety and efficacy of Blue #1 in various studies, we

have developed a technique to measure the concentration of food dyes, including Blue #1, in blood to assess their utility in quantifying intestinal barrier function in humans.

Materials and methods

The study was approved by the institutional review board of the University of California, Los Angeles. All patients or their legal representatives gave written informed consent.

Study design

This study was a prospective single-center study in 14 adult subjects (10 in the intensive care unit [ICU] and four healthy volunteers). Phase 1 of the study recruited healthy volunteers and was designed to establish the lower limit of detection and to measure the baseline intestinal absorption in normal subjects. Phase 2 was performed in 10 critically ill patients recruited in the ICU. Clinically septic subjects were defined by the Surviving Sepsis Campaign (including presence, probable or documented, of infection together with systemic manifestations such as fever, tachycardia, leukocytosis, hypotension, organ dysfunction, or decreased tissue perfusion).[51] Inclusion criteria included English-speaking adult subjects over the age of 18, who were able to tolerate enteral intake and being up at >45° for 1 h after Blue #1 administration. Exclusion criteria included having a strict nothing by mouth (NPO) status, documented history of hypersensitivity to Blue #1, or for Phase 1, having any acute systemic illness.

Subjects were administered 0.5 mg/kg Blue #1 orally or per nasogastric tube as an aqueous solution of diluted food coloring (1.0 mg/mL). Five blood specimens were drawn per subject (5 mL/draw) at 0 h (before dose), 1 h, 2 h, 4 h, and 8 h after dye ingestion.

Detection of dye

Blue #1 presence was assessed in plasma samples using a modified liquid chromatography/tandem mass spectrometry method. In brief, plasma samples were mixed with three volumes of the mixture of isopropanol and acetonitrile (1:3, vol:vol) containing 0.1% of trifluoroacetic acid. After 15 min of incubation at room temperature, samples were spun down using table top centrifuge (16,000 rpm for 5 min) and supernatants transferred to fresh Eppendorf tubes and dried using Speed-Vac. Dried residue was dissolved in 50% N-methyl-2-pyrrolidone in water containing p-nitroanilide of N-benzyl-L-arginine (Bzl-Arg-pNA) as an internal standard. All samples were analyzed using the Agilent 6460 Triple Quadrupole LC-MS System (Agilent Technologies, Santa Clara, CA), equipped with a Cortecs C18+ 2.1 × 50 mm column (Waters Corp, Milford, MA). Mobile phases consisted of solvent A (20 mM $HCOONH_4$, 0.1 % formic acid in water) and solvent B (2 mM $HCOONH_4$, 0.1 % formic acid in mixture of methanol/acetonitrile/water [68:30:2; vol:vol:vol]). The dye was detected in the positive ionization mode using selected reaction monitoring transition (m/z 749.1→170.9) applying the linear gradient of solvent B from 0% to 100% over 10 min (flow rate: 0.2 mL/min).

Statistics and data analysis

All enrolled participants were included in the final analysis. Data on subjects were summarized and compared among the groups. Demographics and baseline characteristics of the subjects were summarized descriptively by means and standard deviations for continuous variables and frequency distribution for categorical variables. Summaries were performed based on all subjects. For correlation analysis, clinical variables and outcomes were used to correlate with the amount of absorbed dye. The differences in detection of Blue #1 between groups were tested using independent samples *t*-test. All statistical analyses were performed using Stata IC 15.0 (StataCorp LLC, College Station, TX). A P value < 0.05 was considered statistically significant.

Results

Between June 1, 2016, and April 15, 2017, 14 subjects (ICU, $n = 10$ *versus* healthy, $n = 4$) were recruited for the study (Table 1). None of the healthy control subjects met sepsis criteria or were on vasopressors. They had all been NPO for at least 12 h before dye administration. Among the subjects in the ICU, four were not classified as septic as they lacked probable or documented evidence of infection. Of these four subjects, one was on vasopressor support to allow for continuous venovenous hemodialysis for aggressive fluid removal. The remaining six subjects met sepsis criteria and were on vasopressors of varying doses in the absence of hypovolemic or cardiogenic etiology.

First, we attempted to ascertain the lower limit of detection, as well as to measure the normal intestinal absorption of Blue #1 in healthy subjects. In all analyzed healthy, ambulatory subjects, there was no detectable absorption of Blue #1 in plasma samples retrieved up to 8 h after dye administration at a dose of 0.5 mg/kg (Fig. 1).

Next, we expanded the study to critically ill subjects in the ICU. Overall, the absorption of Blue #1 was greater in septic patients after 2 h and 8 h of dye administration when compared to nonseptic patients (healthy subjects and critically ill patients not meeting the criteria for septic shock) in which Blue #1 absorption was virtually absent (Fig. 1). This difference was statistically significant at 2 h after dye administration (Table 2).

Similar analyses were performed comparing subjects on or not on vasopressors (Fig. 2). Patients requiring vasopressor support showed greater levels of Blue #1 detection and absorption at 2 h and 8 h after dye administration when compared to healthy subjects and critically ill patients not requiring vasopressor support, though results did not reach statistical significance (Table 2).

Discussion

Current methods of assessing intestinal barrier function and disruption are impractical and costly. Here, we present the first clinical study showing that FD&C Blue #1 can be used as a measure to quantify loss of intestinal barrier integrity as exemplified by the varying degrees of dye absorption. The use of this dye is associated with low cost and the method of detection is feasible to perform in a clinical setting.

In this pilot study, we were able to demonstrate a correlation between sepsis and dye absorption levels. Although there was no statistically significant correlation between dye absorption levels and other factors, such as Model for End-stage Liver Disease (MELD) score, Acute Physiology And Chronic Health Evaluation II (APACHE II) score, age, vasopressor support, or enteric feeding status, the study was not powered to that end. We cannot exclude the possibility that a correlation does exist, in fact, as these variables are themselves highly correlated with critical illness; a larger study may demonstrate this. Currently, we are expanding this study to a larger population of critically ill patients, beyond those with end-stage liver disease, to gain insight into the factors that determine intestinal permeability in those settings.

There have been some concerns with the safety of Blue #1. Though pharmacologic, pharmacokinetic, or toxicologic studies of Blue #1 in humans are incomplete,[47,52] there have been case reports of adverse reactions reported in the literature associated with its use.[49,53–55] Specifically, this dye had been routinely used in large amounts in enteral feeds for the detection of aspiration in the critical care setting.[56] There have been reports of blue discoloration of skin, urine, feces, and serum, as well as development of metabolic acidosis, refractory hypotension, and even death. This is speculated to be due to a dye-mediated dose-dependent inhibition of mitochondrial oxidative phosphorylation, possibly blocking adenine nucleotide translocators. This effect has been observed *in vitro*[57] and can lead to an increased dependence on anaerobic metabolism and a propensity for metabolic acidosis.[53] However, although the dosages used in these reports were all within the approved maximum daily dose, there was significant variability in the administered dosages and Blue #1 was often delivered continuously over multiple days in critically ill patients. The dosage used in our study (0.5 mg/kg) was well below any of those reported in these cases and was given as a one-time bolus. Interestingly, almost all the reported cases involved patients with reported histories of sepsis and presumably with disruption of the intestinal barrier integrity,[53,55,58] pointing to the importance of measuring changes in intestinal absorption in a variety of clinical settings.

In line with published studies reporting the association of impaired intestinal barrier function and sepsis or critical illness,[4,5] we show that enteric absorption of Blue #1 is increased in septic patients and those requiring vasopressor support. The disturbance of intestinal barrier integrity presumably permits translocation of pathogenic elements.[4,8,10–12] This could in turn potentially lead to a vicious cycle of critical illness and exposure and susceptibility to further pathogenic insults. Understanding the pathophysiology of this process with the development of a reliable and feasible method is important to improving the care of critically ill patients.

Conclusions

In summary, our studies demonstrate a novel, easy-to-use method of measuring intestinal barrier integrity utilizing a food grade dye that is detectable by mass spectrometry analysis of patients' blood at multiple time points following enteric administration. This method would allow for the measurement of intestinal barrier function in patients at risk for sepsis, organ failure, or other conditions where loss of integrity of the intestinal barrier could lead to

adverse symptoms or secondary effects. Insight into intestinal barrier integrity and function is important to improve our knowledge of disease etiology and pathophysiology and to contribute to early detection and/or secondary prevention of disease.

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Disclosures

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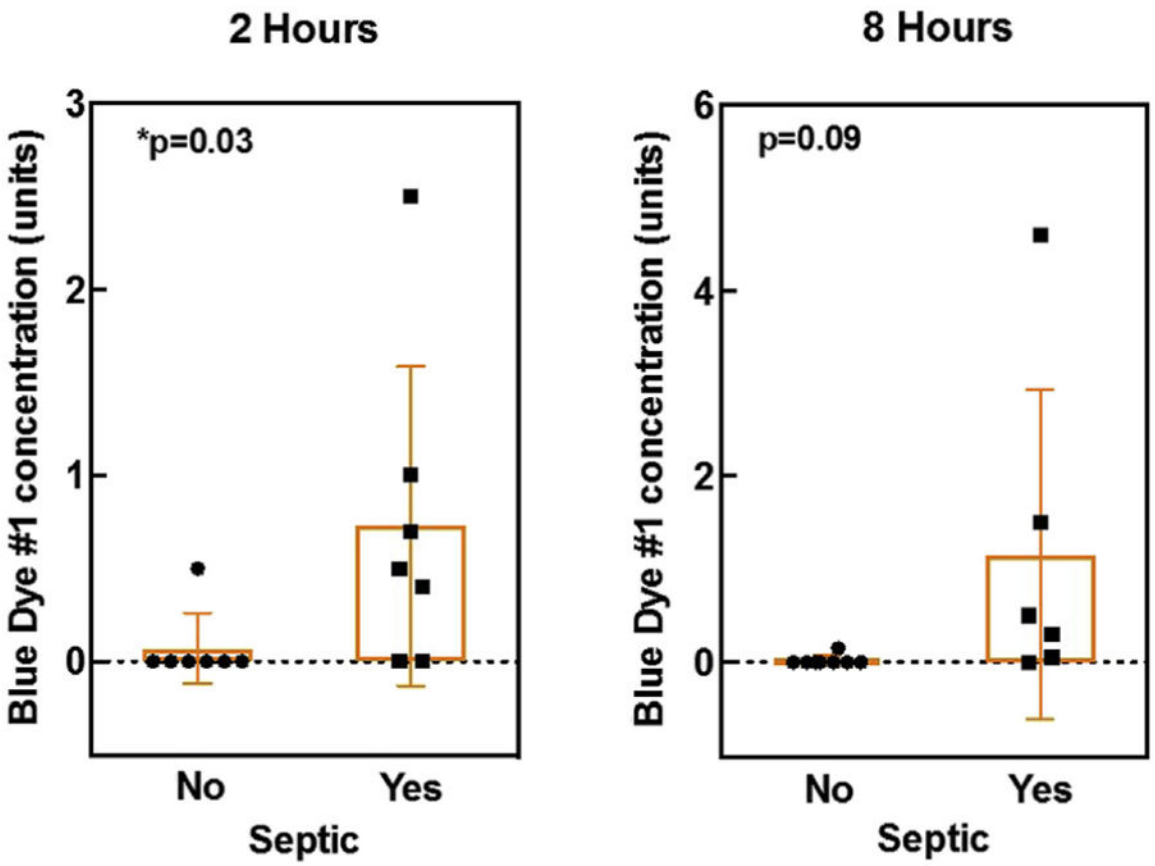

Fig. 1.

Peak height of Blue #1 detected in plasma of nonseptic versus septic subjects at 2 h and 8 h after dye administration. (Color version of figure is available online.)

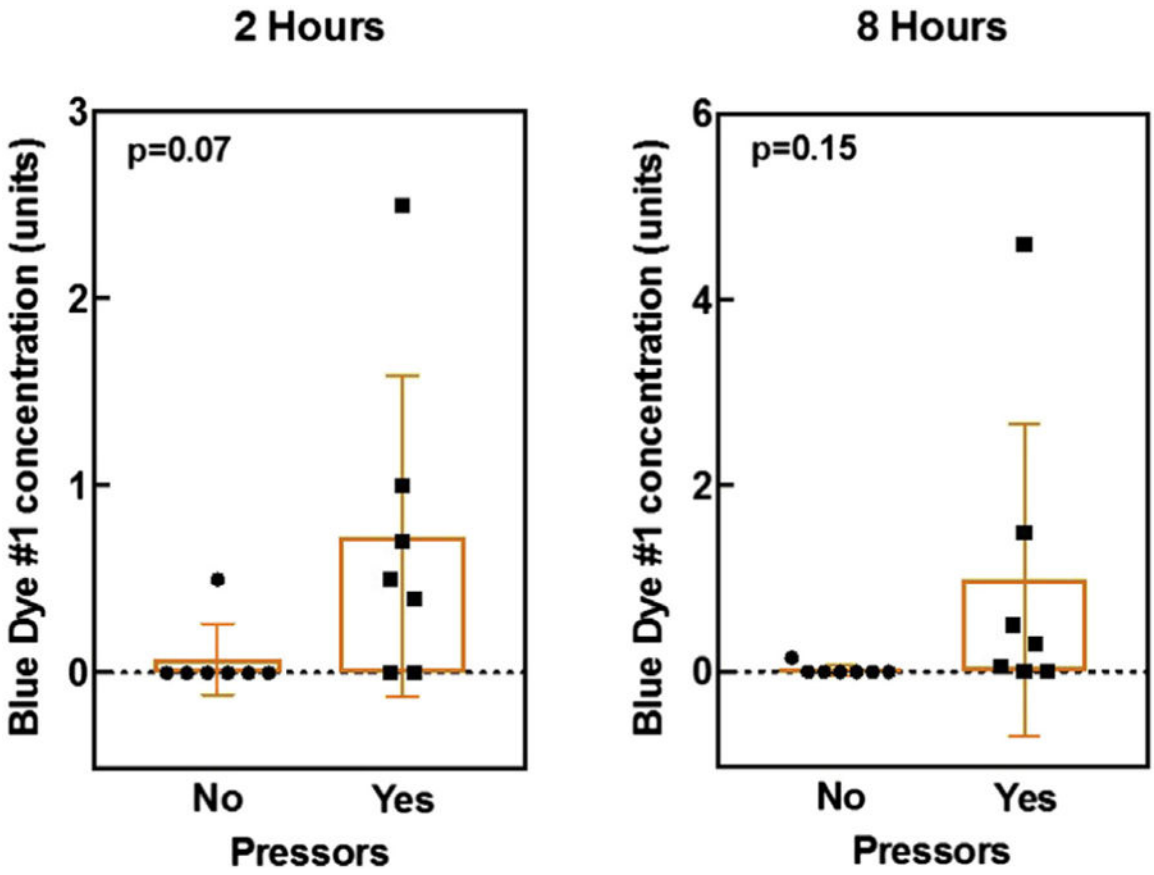

Fig. 2.

Peak height of Blue #1 detected in plasma of subjects without vasopressor support and those on vasopressor support at 2 h and 8 h after dye administration. (Color version of figure is available online.)

Table 1 –

Clinical characteristics of subjects.

Subject	Location	Sepsis criteria met	Vasopressor support	Norepinephrine equivalent dose (mcg/kg/min)	MELD score	By mouth status	APACHE II score	Source of sepsis	2 h dye absorption (peak height)
1	Ambulatory	N	N	0	6	Reg diet	0	N/A	0
2	Ambulatory	N	N	0	6	Reg diet	0	N/A	0
3	Ambulatory	N	N	0	6	Reg diet	0	N/A	0
4	Ambulatory	N	N	0	6	Reg diet	0	N/A	0
5	ICU	Y	Y	0.12	34	NPO × meds	15	Ascites, *E. coli*	0.5
6	ICU	Y	Y	0.12	39	NPO × meds	24	Respiratory, methicillin-resistant *Staphylococcus aureus*	0.7
7	ICU	Y	Y	10.10	35	NPO × meds	16	Respiratory, *Candida*	0
8	ICU	Y	Y	0.25	40	NPO × meds	21	Blood and respiratory, *Candida*	2.5
9	ICU	Y	Y	0.02	23	Reg diet	14	Urine, *enterococcus*	0.4
10	ICU	Y	Y	5.00	43	NPO × meds	21	Blood, *Klebsiella*	1
11	ICU	N	N	0	40	Reg diet	26	N/A	0.5
12	ICU	N	N	0	33	Reg diet	12	N/A	0
13	ICU	N	N	0	17	Reg diet	13	N/A	0
14	ICU	N	Y	0.40	36	Reg diet	20	N/A	0

Table 2 –

Serum blue#1 quantitation.

Time point	Peak height (Mean ± SD)		*P*	Peak height (Mean ± SD)		*P*
	Septic (*n* = 6)	Nonseptic (*n* = 8)		Vasopressor support (*n* = 7)	No vasopressor support (*n* = 7)	
2 h	0.850 ± 0.873	0.0625 ± 0.177	0.03	0.729 ± 0.860	0.071 ± 0.189	0.07
8 h	1.158 ± 1.772	0.019 ± 0.053	0.09	0.993 ± 1.676	0.021 ± 0.056	0.15