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Fluorescence Intensity between Standard versus Diluted Indocyanine Green to Evaluate Flap Perfusion in Rats

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Summary: The ideal dose for indocyanine green (ICG) has not been established yet, although 5 mg per mL is widely accepted for free flap evaluation. Due to its high price and rarity in developing countries, this preliminary study aimed to find the lowest concentration of ICG without reducing the fluorescence quality read by near-infrared camera in animal models. An experimental study was conducted on 25 Wistar rats divided into five groups based on the injected ICG, which was in 5 mg per mL, 3.75 mg per mL, 2.5 mg per mL, 1.25 mg per mL, and 0.5 mg per mL concentrations. The epigastric flap was elevated and confirmed to be vital on the fifth day. Upon confirmation, bolus IV injection of ICG was given via the tail, and the flap was read using near-infrared camera. The 25 different videos are randomized and rated individually in a blind manner by five microsurgeons, chosen beforehand. The videos are evaluated with a scoring system ranging from 0 to 4, assessing fluorescence visibility and flap vasculature. Nonetheless, the intraclass correlation coefficient is 0.779. There was no difference between standard and diluted ICG concentrations to evaluate flap perfusion. The 2.5 mg per mL concentration of ICG was the most favorable. This finding is not clinically relevant for application in human subjects yet. However, this study shows promising results for further usage of ICG in daily practice at a lower cost. *(Plast Reconstr Surg Glob Open 2024; 12:e5948; doi: 10.1097/GOX.0000000000005948; Published online 3 July 2024.)*

INTRODUCTION

The current gold standard for assessing free flap viability relies heavily on subjective clinician evaluations, leading to inconsistent results.[1] Postoperative monitoring involves frequent assessments, initially every 30–60 minutes for 24 hours, then every 1–2 hours for 48 hours, extending to the fifth day. Delegating monitoring exacerbates variability.[2,3]

Indocyanine green (ICG) as vascular imaging shows promise due to its nonradioactive nature, facile excretion via bile, nontoxicity, and intravascular compartment entrapment.[4] However the optimal ICG concentration and safety of repeated administrations remains contentious.[5–7] To date, existing studies have predominantly presented expert consensus on ICG dosage without experimental evidence or rationale elucidating the superiority of specific concentrations over others in scientific literature.[8,9] The traditional near-infrared radiation (NIR) cameras are usually used for evaluating emitted fluorescence and more cost-effective alternatives are needed due to the high cost of ICG and NIR cameras.[10] This approach aims to improve flap perfusion assessment while considering cost and safety concerns of conventional methods.

METHODS

This animal study involved five groups, each comprising five rats, receiving varying ICG, Aurogreen (Aurolab, Tamil Nadu, India), concentrations: 5, 3.75, 2.5, 1.25, and 0.5 mg per mL, approved by the ethics board. The Federer formula determined a sample size of 25 rats.

Subsequently, epigastric pedicle flaps were harvested from the left abdominal region of the rats. ICG was injected intravenously via tail on the fifth day postharvest, and fluorescence was captured using an NIR camera

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Received for publication February 20, 2024; accepted May 10, 2024.

DOI: 10.1097/GOX.0000000000005948

Disclosure statements are at the end of this article, following the correspondence information.

Related Digital Media are available in the full-text version of the article on www.PRSGlobalOpen.com.

(Fluoro 4000XL, Tohaoptics, Japan) 10–40 cm from the flap under controlled lighting conditions.[11]

Five assessors independently assessed the visual changes by scoring the fluorescence clarity and flap vasculature in videos, using a scale from 0 to 4. A control video was shown intermittently. Assessors only had 20 seconds per video [**See Video (online),** which displays the **c**omparison of ICG fluorescence in a viable flap across concentrations. Note: a. 5 mg/mL concentration, b. 3.75 mg/mL concentration, c. 2.5 mg/mL concentration, d. 1.75 mg/mL concentration, and e. 0.5 mg/mL concentration], and subjectivity is acknowledged. Data analysis utilized SPSS version 24.0, using one-way ANOVA or the Kruskal-Wallis test ($\alpha = 0.05$).

Takeaways

Question: How to achieve the lowest concentration of indocyanine green (ICG) without reducing the fluorescence quality?

Findings: The utilization of ICG at a concentration of 2.5 mg per mL can be considered a feasible substitute for standard concentration to evaluate the flap vasculature in rats despite a statistically nonsignificant difference between the fluorescence intensity at 5 mg per mL ICG concentration and the diluted ICG concentrations.

Meaning: The 2.5 mg per mL concentration of ICG is the most favorable concentration in rats for optimal flap perfusion visualization. This study shows promising results for further usage of ICG in daily practice at a lower cost.

RESULTS

Twenty-five male Wistar rats, weighing between 265 and 325 g, had their vital epigastric flaps assessed in offline sessions, with one evaluator conducting online assessments. Descriptive analysis of nonnormally distributed data highlighted varying mean scores, where the 2.5 mg per mL concentration yielded the highest (2.800), followed by 5 mg per mL (2.560), 1.25 mg per mL (2.520), 3.75 mg per mL (2.160), and 0.5 mg per mL (1.960), as outlined in Supplemental Digital Content 1 and Video 1. (**See table, Supplemental Digital Content 1,** which displays cumulative scoring from the assessors. **http://links.lww.com/PRSGO/D336**) [**See Video (online),** which displays the comparison of ICG fluorescence in a viable flap across concentrations. Note: a. 5 mg per mL concentration, b. 3.75 mg per mL concentration, c. 2.5 mg per mL concentration, d. 1.75 mg per mL concentration, and e. 0.5 mg per mL concentration.] The ICG fluorescence difference between 5 mg per mL and 2.5 mg per mL can be seen in Figure 1. The intraclass correlation coefficient was computed to ascertain interrater reliability among assessors as per the Landis and Koch classification with average measure of 0.779 (95% CI, 0.605–0.890; $P < 0.001$).

Upon statistical analysis, the values for concentrations of 5 mg per mL, 2.5 mg per mL, and 1.25 mg per mL were 3 (1–4). Conversely, concentrations of 3.75 mg per mL and 0.5 mg per mL were 2 (1–4). Kruskal-Wallis test disclosed a significant statistical disparity across at least one pair of concentration groups. Post hoc Mann-Whitney tests indicated no significant statistical difference between standard (5 mg/mL) and diluted ICG concentrations ($P > 0.05$), as detailed in Supplemental Digital Content 2. (**See table, Supplemental Digital Content 2,** which displays comparison of standard and diluted concentrations of indocyanine green in evaluating flap perfusion. **http://links.lww.com/PRSGO/D337**.)

DISCUSSION

Demographic Characteristics of Subject

The subject's body weight significantly impacts total blood volume and fluorescence intensity. Wistar rats typically have a total blood volume of about 64 mL per kg, varying with weight.[12] In this study, rats weighed 265–325 g, with total blood volumes around 16.9–20.8 mL. Blood volume changes affect ICG fluorescence by altering blood protein, erythrocyte, and leukocyte levels. Higher counts of erythrocytes and leukocytes may hinder fluorescence, whereas increased plasma proteins could enhance it. Therefore, precise understanding of subject characteristics, especially body weight and related physiological factors, is crucial for interpreting fluorescence data accurately.[7,10]

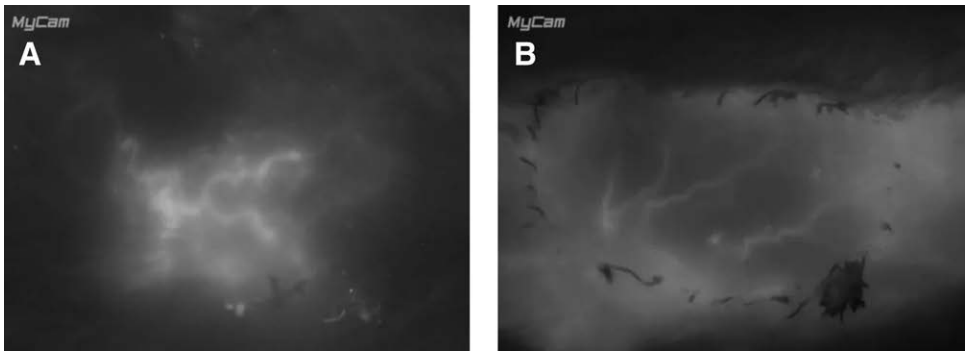

Fig. 1. ICG fluorescence evaluation in rats. Schematic ICG fluorescence to evaluate flap perfusion can be seen in the 5mg per mL concentration (A), showing an average score of 2.560, and the 2.5mg per mL concentration (B), showing an average score of 2.800. The optimal fluorescence is seen in 2.5mg per mL concentration.

The Measuring Device

An NIR camera, equipped with light-emitting diode lights emitting radiation at a wavelength of 760 nm. This study encompassed distances ranging from 10 to 40 cm between the camera and the subject, corresponding to light intensities measured at 12–4 mW, respectively. Optimal fluorescence of ICG is observed within a wavelength range of 760–785 nm, eliciting fluorescence production within the range of 820–849 nm.[11] The standardization of camera usage across subjects serves to mitigate potential confounding variables and ensures consistency in each procedure.

ICG Pharmaceutical Properties

ICG elicits fluorescence upon binding with plasmatic protein or lipoprotein.[7] It tends to aggregate based on concentration and solvent. Concentrations over 100 µM often lead to aggregation into oligomeric structures, thereby diminishing fluorescence intensity. Moreover, a quenching effect is observed at higher ICG concentrations, thus reducing fluorescence intensity, noticeable in vivo above 0.1 mg per mL.[10] This effect, along with aggregation and protein binding, affects ICG fluorescence.[7,10]

Statistical analysis indicates no significant difference in fluorescence intensity between 5 mg per mL ICG and diluted variants. Optimal concentrations for visualizing flap vasculature are 2.5 mg per mL, 5 mg per mL, and 1.25 mg per mL, respectively. At 2.5 mg per mL, equilibrium optimizes dosage and protein binding, enhancing fluorescence. Concentrations below 2.5 mg per mL weaken intensity, whereas higher ones may intensify quenching and aggregation. Notably, 3.75 mg per mL and 0.5 mg per mL concentrations yield unsatisfactory results, deviating from expectations. At the 3.75 mg per mL concentration, deviation from expected outcomes was observed. Our hypothesis suggested that higher concentrations would induce greater polymerization of ICG, thus diminishing fluorescence quality. The presence of more ICG polymers and fewer ICG bonding with plasma protein (quenching phenomenon) at 3.75 mg per mL compared with 5 mg per mL led to reduced fluorescence. This finding aligns with Desmettre et al, indicating nonlinearity between ICG concentration and fluorescence.[12] The 0.5 mg per mL concentration limits ICG accessibility, resulting in weak fluorescence due to poor skin penetration. Although 2.5 mg per mL ICG is practical for rat models, its generalizability to humans is limited. Study limitations include findings not directly applicable to clinical practice and the subjective nature of ICG fluorescence evaluation. Determining visually superior concentrations was inconclusive due to the lack of direct causative analysis. Nonetheless, these insights are valuable for future research, particularly in developing countries relying on ICG for evaluating tissue flaps in humans.

CONCLUSIONS

Our study reveals optimal flap perfusion assessment in rats with 2.5 mg per mL ICG angiography, despite statistically nonsignificant differences among concentrations. Although not directly translatable to humans, these findings offer valuable insights for cost-effective ICG utilization in clinical practice.

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DISCLOSURE

The authors have no financial interest to declare in relation to the content of this article.

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