Enhanced-Reality Video Fluorescence

A Real-Time Assessment of Intestinal Viability

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Objective: Our aim was to evaluate a fluorescence-based enhanced-reality system to assess intestinal viability in a laparoscopic mesenteric ischemia model.

Materials and Methods: A small bowel loop was exposed, and 3 to 4 mesenteric vessels were clipped in 6 pigs. Indocyanine green (ICG) was administered intravenously 15 minutes later. The bowel was illuminated with an incoherent light source laparoscope (D-light-P, KarlStorz). The ICG fluorescence signal was analyzed with Ad Hoc imaging software (VR-RENDER), which provides a digital perfusion cartography that was superimposed to the intraoperative laparoscopic image [augmented reality (AR) synthesis]. Five regions of interest (ROIs) were marked under AR guidance (1, 2a-2b, 3a-3b corresponding to the ischemic, marginal, and vascularized zones, respectively). One hour later, capillary blood samples were obtained by puncturing the bowel serosa at the identified ROIs and lactates were measured using the EDGE analyzer. A surgical biopsy of each intestinal ROI was sent for mitochondrial respiratory rate assessment and for metabolites quantification.

Results: Mean capillary lactate levels were 3.98 (SD = 1.91) versus 1.05 (SD = 0.46) versus 0.74 (SD = 0.34) mmol/L at ROI 1 versus 2a-2b (P = 0.0001) versus 3a-3b (P = 0.0001), respectively. Mean maximal mitochondrial respiratory rate was 104.4 (±21.58) pmolO_2/second/mg at the ROI 1 versus 191.1 ± 14.48 (2b, P = 0.03) versus 180.4 ± 16.71 (3a, P = 0.02) versus 199.2 ± 25.21 (3b, P = 0.02). Alanine, choline, ethanolamine, glucose, lactate, myoinositol, phosphocholine, sylloinositol, and valine showed statistically significant different concentrations between ischemic and nonischemic segments.

Conclusions: Fluorescence-based AR may effectively detect the boundary between the ischemic and the vascularized zones in this experimental model.

Keywords: augmented reality, capillary lactate, indocyanine green (ICG) video fluorescence, intestinal viability, ischemia, laparoscopic intraoperative ischemia assessment, metabonomics, mitochondrial, nuclear magnetic resonance, oxidative stress, respiration, spectroscopy.

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Sufficient blood supply is of paramount importance for successful anastomotic healing and avoidance of intestinal ischemia and necrosis. Insufficient microcirculation of the anastomosed segments leads to anastomotic leakage and stricture.[1] Intestinal microcirculation and viability are usually estimated from the color of the serosa's surface, the presence of peristalsis, pulsation, and bleeding from marginal arteries. This is subjective and based on the surgeon's experience and may well lead to misinterpretations.[2] Several methods have been proposed to assess the intestinal viability intraoperatively and guide the extent of the resection.[3] The most reliable techniques include spectroscopy and fluorescence methods.

Spectroscopic studies, near-infrared spectroscopy (NIRS) and visible light spectroscopy (VLS), evaluate O_2 tension (StO_2) by assessing the amount of light passing through tissues. On the basis of wavelength, NIRS (700–730 nm) is suitable for all vascular segments whereas VLS (475–625 nm) is suitable mainly for capillaries. In a series of 20 patients undergoing colorectal resection, Hirano et al[4] showed that StO_2 can be safely and reliably measured by NIRS and that a low StO_2 is strongly related to an increased risk of anastomotic leakage. An important limitation of the StO_2 method is the lack of uniformity in StO_2 measurements between various VLS and NIRS equipment and different wavelength profiles.

Fluorescence studies, perfusion fluorometry or laser fluorescence angiography (LFA), evaluate blood flow by referring to the fluorescence of a dye [fluorescein or indocyanine green (ICG)] when illuminated by a laser.

Kudszus et al[5] recently evaluated the impact of indocyanine-green LFA in a case-matched study involving 201 patients undergoing open colorectal resection with LFA compared with a retrospective well-matched cohort of 201 patients. Authors found that LFA may significantly reduce the rate of severe complications and the rate of anastomotic leakage.

In 2011, Matsui and Winer evaluated the ability of intraoperative near-infrared (NIR) ICG–based angiography to predict ischemic bowel in an experimental model of mesenteric ischemia in pigs and rats. Authors used the FLARE (Fluorescence-Assisted Resection and Exploration) imaging system, consisting in light-emitting diodes in the spectra of white light (400–650 nm) and NIR fluorescence excitation light (745–779 nm). The exposed bowel images were acquired using customary optics and eventually merged with the ICG fluorescence distribution images. The FLARE demonstrated better accuracy in predicting bowel survival when compared with clinical evaluation alone.[6]

The use of this technology has been limited to open surgery, and new tools are becoming available to extend the benefits of fluorescence-guided surgery to the minimally invasive approach.[7–9]

Recently, a new incoherent light source (D-light P) equipped with a special filter and special NIR-optimized endoscope to detect the ICG fluorescence signal has been developed by Karl Storz, Tüttlingen, Germany. Our Research and Development department developed the Ad Hoc imaging software (VRRENDER PERFUSION,

IRCAD) to analyze the region of interest (ROI) within the operative field of the high definition laparoscopic camera, extrapolating the tissue perfusion difference by comparing it with a control area. Fluorescence intensity (as time to fluorescence peak) is directly correlated to perfusion, and the software allows to construct a virtual cartography of the perfused area and to overlay it to the laparoscopic image of the patient. This overlaying of real and synthetic images to enhance the definition of anatomic details goes along with the concept of augmented reality (AR).

The aim of this experimental study was to assess the performance and accuracy of a fluorescence-based AR method to evaluate the intestinal perfusion in a model of mesenteric ischemia by comparing the results of the image analyzer software to the objective measurement methods of visceral and cellular ischemic suffering, local capillary lactate level, mitochondrial activity, and the metabonomic fingerprint of ischemia, at the different ROI in the ischemic and nonischemic bowel.

Lactate is the end product of the anaerobic energetic metabolism acting as a biomarker of tissue hypoxia-ischemia. In this experimental study, we aimed to evaluate the capillary lactate level at the small bowel ROIs outlined by the AR. In addition, we measured mitochondrial activity at the ROIs identified by the AR because this is highly dependent on the O_2 supply and reflects cellular energetic status. Finally, the metabonomic profile of ischemia was defined on the ischemic and nonischemic bowel segments as identified with the AR. The "metabolome" or "metabonome" is the complete set of the low-molecular-weight (<1500 Daltons) metabolites found in a system (cell, tissue, or organism) in a specific condition.[10] Assessing the metabonomic profile implies quantifying the dynamic metabolic response of the living system to physiologic or pathologic stimulation.

MATERIALS AND METHODS

Animals

A total of 7 adult swine were used in this nonsurvival study. One pig was involved in the pilot study to establish the technical steps of the experiment and to assess the ICG dose that produces optimal signals for the image analyzer software. Six animals [3 males and 3 females, mean weight 38.3 kg (SD = 10.2)] were involved in the study protocol.

All animals used in the experimental laboratory were managed according to French laws for animal use and care and according to the directives of the European Community Council (number 86/609/EEC). Pigs were fasted for 24 hours before surgery with free access to water. Premedication by intramuscular injection of ketamine (20 mg/kg) and azaperone (2 mg/kg) (Stresnil, Janssen-Cilag, Belgium) was administered 1 hour before surgery. Induction was achieved by intravenous propofol (3 mg/kg) combined with pancuronium (0.2 mg/kg). Anesthesia was maintained with 2% isoflurane. At the end of the procedure, the pig was sacrificed according to our protocol with an endovenous injection of a lethal dose of potassium chloride.

NIR Optimized Laparoscope

To capture the fluorescence signal, the D-light P laparoscope (Karl Storz, Tüttlingen, Germany) was used. The filter of the D-light P can be switched into the light transmission path. The filter transmits light in a range of 690 to 780 nm and completely blocks light of more than 800 nm. In addition, switching the filter out of the light path allows standard white light observation.

Image Analyzer Software

VR RENDER is a proprietary medical imaging analyzer software suite, which allows for 3D reconstruction of Digital Imaging and Communication in Medicine (DICOM) images.

VR-RENDER PERFUSION is a plug-in to the VR RENDER using the Python Scientific Package, with a modified algorithm able to correlate fluorescence over time intensity changes to tissue perfusion.

Determination of the Optimal ICG Dose Regimen

The minimum dose regimen of ICG to obtain a reliable fluorescence that could be converted into a pixel/intensity curve using the VR-RENDER PERFUSION software was determined in a pilot pig in which 4 different doses of ICG—0.05 mg/kg, 0.12 mg/kg, 0.25 mg/kg, and 0.5 mg/kg—were injected intravenously after creation of 4 ischemic small bowel loops. Digital videos were recorded and analyzed with the software during the procedure to determine the dose of ICG allowing for an optimal signal/noise ratio.

Laparoscopic Creation of the Model of Ischemia

A pneumoperitoneum was established with a Veress needle. A vertical midline incision was made and a 10-mm port was introduced through the fascia for the D-Light-P laparoscope (Karl Storz, Tüttlingen, Germany). Two additional 10-mm working ports were placed. A loop of small bowel was identified and suspended using 3 transparietal fixing devices (T-Pea Lifter, Surgical Perspective, France). Windows were created in the mesentery and 3 to 4 vessels were clipped. The laparoscope was fixed to an articulated mechanical support to stabilize the image during video capture. After 15 minutes of ischemia, the scope was shifted to laser modality and 0.5 mg/kg of ICG (ICG-PULSION, Pulsion Medical System, Munich, Germany) was rapidly injected intravenously. Ventilation was suppressed for 20 seconds to stop the breathing motion during video capture.

Augmented Reality Identification of the Ischemic Area

The algorithm of the VR-RENDER PERFUSION highlighted the signals generated at maximum fluorescence intensity, at the starting flow point, and at the time to reach peak level. The software was set to detect areas of the bowel in which there was a delay of at least 50% to reach peak of fluorescence intensity. This allowed to construct digital cartography with a code-color correlated to the intestinal perfusion, which was overlapped to the laparoscopic real-time images to obtain AR. The ROIs were marked laparoscopically on the small bowel serosa using a surgical pen mounted over a needle holder and following the profile designed by the AR (Fig. 1). The ROIs were coded as follows: segment 1 (ischemic area); segments 2a and 2b (marginal areas or future resection lines); segments 3a and 3b (vascular areas) (see Supplemental Digital Content 1, which shows the procedure, at http://links.lww.com/SLA/A370).

Systemic and Local Capillary Lactates Measurement

After 60 minutes of ischemia, a laparotomy was performed and the small bowel was exteriorized. Systemic capillary lactates were measured on a blood sample obtained by puncturing the groin of the pig, and local capillary lactates were measured on blood samples obtained by puncturing the serosa at the ROI outlined by the AR using a handheld device (EDGE analyzer, Apex Biotechnology, Taiwan). The order of sampling at the ROI was predetermined by means of randomizer software.

Mitochondrial Respiratory Chain Assessment

Full-thickness bowel biopsies were taken at the previously marked ROI following the same predetermined randomized order.

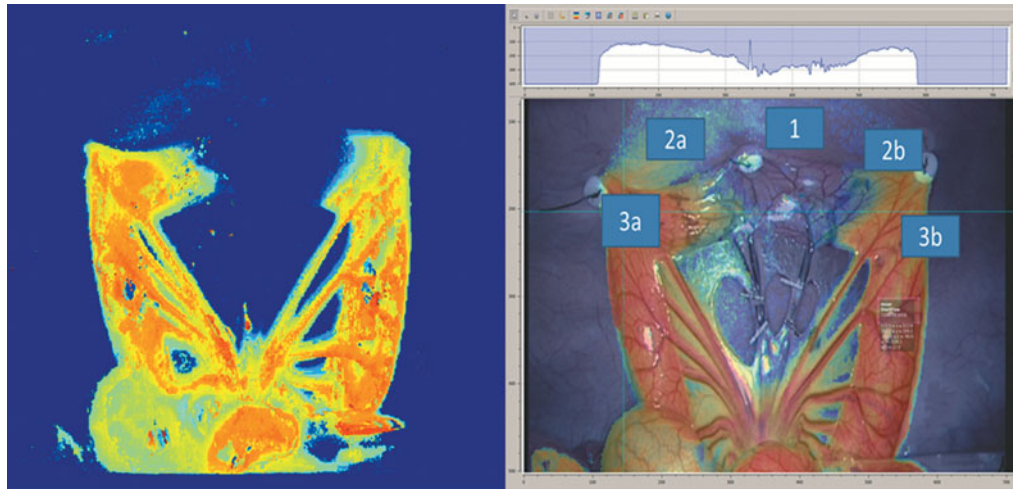

FIGURE 1. Augmented reality identification of the ischemic area. VR RENDER PERFUSION software correlated fluorescence intensity changes over time to the tissue perfusion. The algorithm highlighted the signals generated at maximum fluorescence intensity, at the starting flow point, and at the time to reach peak level. This allowed to put together a digital cartography correlated to the intestinal perfusion, which was overlapped to the real-time laparoscopic images. The ROIs were marked laparoscopically on the small bowel serosa using a surgical pen mounted over a needle holder and following the profile designed by the fluorescence-based augmented reality. The ROIs were coded as follows: segment 1 (ischemic area), segments 2a and 2b (marginal areas), segments 3a and 3b (vascular areas).

Mitochondrial activity was determined as previously reported.[11] Intestinal biopsies were quickly cut into 5 pieces each (1–2 mm^3) allowing mitochondria challenge within their cellular environment. The samples were placed in a 2 mL water-jacketed oxygraphic cell (Oxygraph-2k, Oroboros instruments, Innsbrück, Austria) equipped with a Clark electrode. After determination of basal oxygen consumption (V0), the maximal tissue respiration rates were measured at 37°C under continuous stirring in the presence of saturating amount of adenosine triphosphate as phosphate acceptor (V max). Once V max has been recorded, electron flow goes through complexes I, III, and IV, because of the presence of glutamate (5 mmol/L) and malate (2 mmol/L). Complex I was then blocked with Amytal (0.02 mmol/L) and complex II was stimulated with succinate (25 mmol/L). In this condition, mitochondrial respiration is evaluated by complexes II, III, IV (V_{succ}). Finally, N,N,N',N'-tetramethyl-p-phenylenediamine dihydrochloride (TMPD, 0.5 mmol/L) and ascorbate (0.5 mmol/L) were added as an artificial electron donor to cytochrome c. So, cytochrome c oxidase (complex IV) is studied as an isolated step of respiratory chain (V_{tmpd}).

After the experiments, tissues were harvested and dried (15 minutes at 150°C) and respiration rates were expressed as pmol of oxygen/second/mg of dry weight.

Magnetic Resonance Spectroscopy Metabonomics Measurement

A small quantity of the same full-thickness small bowel biopsies taken at the ROI following the predetermined randomized order was immediately snapped frozen in liquid nitrogen to stop any metabolic activity and sent for magnetic resonance spectroscopy. High-resolution magic-angle spinning (HRMAS) spectra were recorded on a Bruker Advance III 500 spectrometer (Bruker Biospin, Fällanden, Switzerland) operating at a proton frequency of 500.13 MHz. The spectrometer is equipped with a 4-mm double-resonance (1H, 13C) gradient HRMAS probe. A Bruker Cooling Unit regulates the temperature at 4°C. All nuclear magnetic resonance (NMR) experiments were conducted on samples spinning at 3502 Hz. For each biopsy sample, a 1D 1H spectrum using a Carr-Purcell-Meiboom-Gill (CPMG) pulse sequence was acquired as previously reported.[12] Metabolites were quantified using the 1D CPMG spectra. Spectra were normalized according to each sample weight and calibrated using the signal intensity of a reference solution containing a known amount of lactate.[13] Peak area integration was used to calculate the concentration. For our experiments, only peaks that were well resolved in 1D CPMG spectra were quantified using MatLab R2010 (MathWorks, France).

Statistical Analysis

Statistics were performed using the GraphPad Prism 5 software. The *t* test or the analysis of variance analyses followed by the Dunnet Multiple Comparison test were used to calculate *P* values for continuous variables, when appropriate. A nonparametric Spearman correlation was also performed when required. A $P < 0.05$ was considered as statistically significant.

RESULTS

The optimal dose of ICG giving the best signal/noise ratio using the setting D-Light (Karl Storz) and the customary VR-RENDER plug-in was determined at 0.5 mg/kg in the pilot study. The surgical procedure was uneventful. Establishment of small bowel ischemia was systematically reproduced in all animals.

Mean *fluorescence time-to-peak* in segment 1 (= ischemic area) was 12.89 seconds (SD = 7.02) and was significantly longer when compared with segments 2a + 2b (= future resection lines) in which it was 6.01 seconds (SD = 3.44), $P = 0.01$, and to segments 3a + 3b (= vascular zones) in which it was 5.69 seconds (SD = 3.68), $P = 0.01$. There was no statistically significant difference between marginal (2a + 2b) and vascular (3a + 3b) zones.

Mean *systemic lactate* level was 0.95 mmol/L (SD = 0.5). Mean *local capillary lactates levels* were statistically significantly higher in the segment 1 (3.98 mmol/L; SD = 1.91) than in segments 2a-2b (1.05 mmol/L; SD = 0.46) and segments 3a-3b (0.74 mmol/LSD 0.34). The nonparametric Spearman correlation showed

a correct relationship between local lactates and fluorescence time-to-peak in the ischemic area ($\rho = 0.71$) and a decreasing correlation in future resection lines ($\rho = 0.5$ for 2a and $\rho = 0.42$ for 2b) and in vascular areas ($\rho = 0.33$ and 0.30).

Mitochondrial Respiratory Chain

Mean V_0 (basal oxygen consumption) in the ischemic area (segment 1) was 66.4 pmol O_2/s/mg (SD = 25.67), and as a result lower when compared with the mean values of all the other segments, reaching a statistically significant difference when compared with the mean values of segment 3a (110 pmol O_2/s/mg; SD = 24.68).

Mean V_{max} (complexes I, III, IV) was statistically significantly reduced in the ischemic zone (104.4 pmol O_2/s/mg of dry tissue; SD = 52.87) when compared with resection line 2b (191.1 pmol O_2/s/mg of dry tissue; SD = 25.08) ($P = 0.03$) and to both perfused zones 3a (180.4 pmol O_2/s/mg of dry tissue; SD = 37.36) ($P = 0.02$) and 3b (199.2 pmol O_2/s/mg of dry tissue; SD = 50.41) ($P = 0.02$).

Mean V_{succi} (complexes II, III, IV) was statistically significantly reduced in the ischemic zone (105.3 pmol O_2/s/mg of dry tissue; SD = 58.14) when compared with zones 2b (213.2 pmol O_2/s/mg of dry tissue; SD = 22.75) ($P = 0.02$) and 3b (219.1 pmol O_2/s/mg of dry tissue SD = 63.86) ($P = 0.01$).

Mean V_{tmpd} (complex IV) was lower in the ischemic zone when compared with both resection lines and vascular zones but the difference was not statistically significant.

Metabonomics

A total of 23 metabolites were quantified in the small bowel biopsies by the NMR HRMAS:(1) lactate, (2) glucose, (3) acetate, (4) alanine, (5) ascorbic acid, (6) asparagine, (7) aspartate, (8) choline, (9) creatinine, (10) ethanolamine, (11) glutamine, (12) glutamate, (13) glutathione, (14) glycerophosphocholine, (15) glycine, (16) lysine, (17) myoinositol, (18) phenylalanine, (19) phosphocholine, (20) sylloinositol, (21) taurine, (22) tyrosine, and (23) valine. For the remaining metabolites, quantification was compromised because of low signals and/or overlapping of the spectra. Among these metabolites, statistically significant different concentrations (expressed in nmol/mg of tissue) between the ROIs 2a, 2b, 3a, and 3b when compared with the ischemic segment 1 were found for alanine, choline, ethanolamine, glucose, lactate, myoinositol, sylloinositol and valine (Fig. 2). No statistically significant differences in concentrations were found between segments 2a or 2b (resection lines) versus 3a or 3b (vascular zones).

DISCUSSION

Proper evaluation of bowel perfusion is the key to success for resection anastomoses in surgery of the gastrointestinal tract. Indeed, improvement in local perfusion due to the development of collateral circulation is unlikely to occur during the first 5 days after a bowel resection with inadequately vascularized margins.[14] Consequently, anastomotic perfusion and intestinal viability are determined at the time of surgery and should be assessed correctly during the surgical procedure. Implementation in the clinical routine of an objective and accurate assessment method of intestinal blood supply could be desirable to decrease the rate of anastomotic complications directly ascribable to reduced perfusion.

Fluorescence-based methods are the most promising, and new platforms are now available for intraoperative assessment in the setting of minimally invasive laparoscopic surgery. However, interpretation of the fluorescence signal in NIR video mode without any image manipulation is difficult. In the present experimental study, we propose a new fluorescence videography-based real-time AR tool to guide the intestinal resection and to assess vascular supply at the future anastomotic site.

Surgical AR is the process of superimposing virtual images of a patient to real-time intraoperative images to "augment" the live view. Virtual images are generally generated by software manipulation of preoperative DICOM images (CT-SCAN, NMR, or US) to obtain a virtual 3D model of the patient. Currently, the virtual model allows surgeons to navigate into the patient's anatomy and plan the surgical procedure. More recent developments aim at integrating the model in the real intraoperative images allowing to guide the surgical procedure by precise discrimination of crucial anatomical details and organ relationships.[15,16] One of the main limitations of AR is in that the virtual model is a snapshot of the preoperative patient anatomy and is not "flexible," and hence the very low intraoperative accuracy of the AR for mobile structures. In our method of assessment of bowel vascular supply, virtual images are directly generated from the intraoperative "live" image signals produced by the specific laparoscope and light source. The system provides an "intraoperative real-time" virtual digital cartography of bowel vascularization on the basis of the fluorescence signal of ICG, which is directly correlated to perfusion.

ICG is a fluorescent dye, which is used to assess cardiac function and liver function and is a well-tolerated compound with some anaphylactic or urticarial reactions described. The ICG binds totally to albumin and solely stays in the intravascular compartment. The uptake of ICG is an active process, and more than 95% of ICG passes in the bile in 15 minutes provided that hepatic function is normal. The current dose of ICG to determine liver function in humans is 0.5 mg/kg.[17] The optimal dose of ICG in fluorescent studies has not yet been determined. Paschen and Muller[18] evaluated the pharmacokinetic of ICG in pigs using the standard dose of 0.5 mg/kg to evaluate liver blood flow. In our pilot study, the dose providing the optimal signal/noise ratio was also 0.5 mg/kg.

In 2006, Toens et al validated the indocyanine (IC) view videography in a rabbit model of ischemia. Animals were injected with ICG (ICG-PULSION, Pulsion Medical System, Munich, Germany) at 0.25 mg/kg. The regions of interest were illuminated with a laser (0.16 w; wavelength 780 nm). Digital videos were recorded using a digital video camcorder (IC View System, Pulsion Medical System, Munich, Germany), showing uptake, steady-state distribution, and substance clearance. Recorded fluorescence was correlated to tissue perfusion using the specific software (IC-CALC SOFTWARE, Pulsion Medical System, Munich, Germany). The increment of fluorescence (pixel intensity) in regions of interest was measured as the curve steepness of light-emission pixel intensity per second.[19] The accuracy of the IC view in assessing intestinal viability was very high.

The main differences between the Toens et al's study and our model are as follows: first, we used a laparoscopic platform equipped with a NIR optical system and second, we converted the fluorescence signal into a digital cartography that was overlapped to the live laparoscopic images to guide resection according to perfusion degree. A third major difference lies in the fact that we did not use fluorescence intensity, which is influenced by the distance between light source and target, but fluorescence time-to-peak is independent of the distance and is related to the blood flow, and gives a dynamic information as opposed to fluorescence intensity alone. The software's algorithm was initially set to discriminate a difference of 50% in fluorescence intensity between the ROIs when compared to the manufacturer's standard, which is a "calibration aid" provided by Pulsion Medical consisting in a sterile plastic card with a squared spot that gives a constant signal when illuminated by NIRS (Fig. 3). After the pilot pig was used, the software setting was changed to identify a 50% rise in time-to-peak (perfusion delay) and alter the color code attributing an "empty spot" to the digital perfusion cartography when the rise in the fluorescence signal was delayed by more than 50%. With this setting, fluorescence-based AR videography allowed to precisely identify the ischemic region and the well-vascularized bowel in our model. The

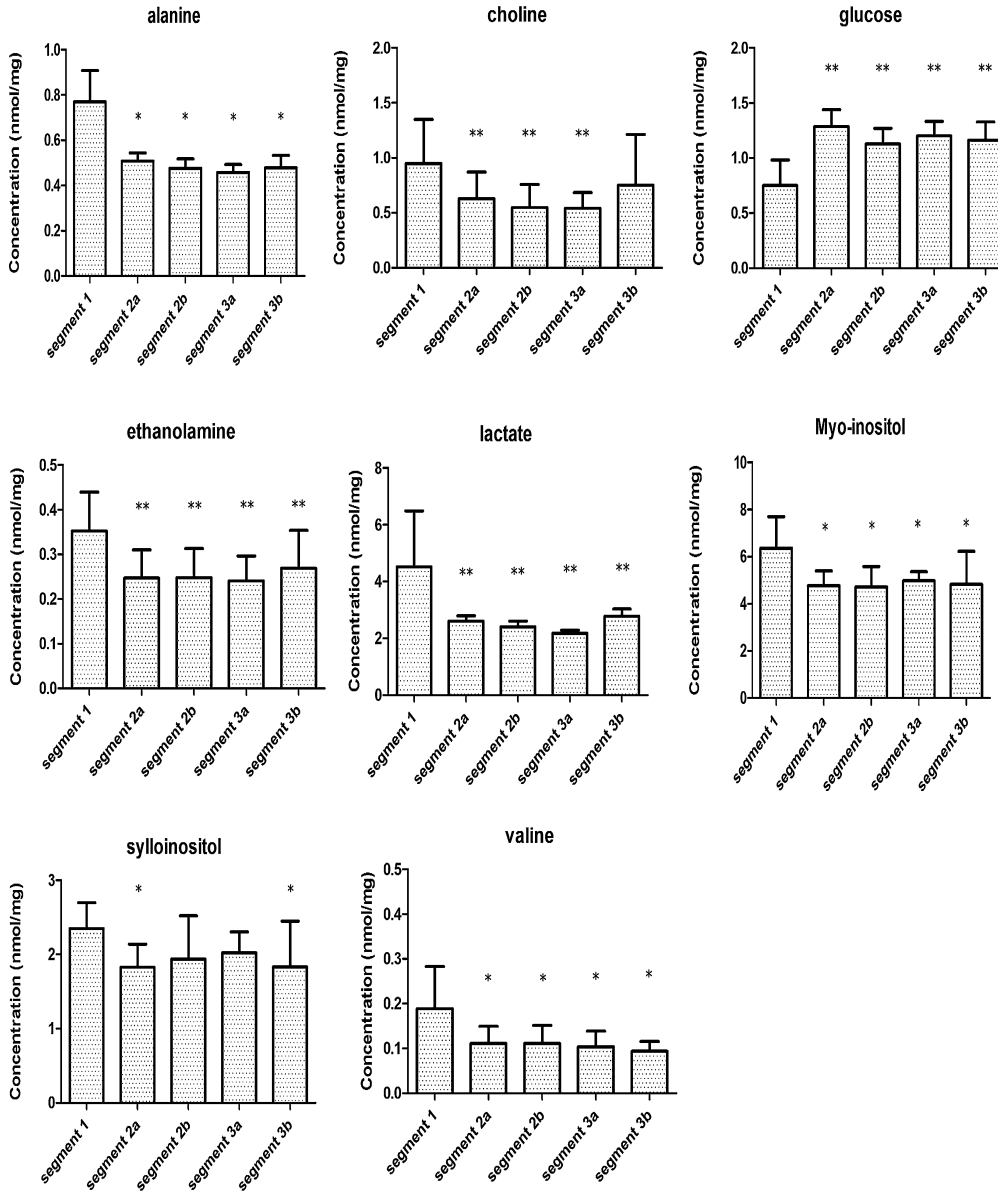

FIGURE 2. Metabolites presenting statistically significant different concentrations between the ischemic (= segment 1) resection lines (= segment 2a-2b) and vascular zones (= segment 3a-3b). Metabolites concentrations are expressed as nmol/mg of tissue. Analysis of variance test followed by Dunnett's test comparing the metabolites concentration in the ischemic segment (segment 1) with segments 2a, 2b, 3a, and 3b. $P < 0.05$ was considered statistically significant. $^{*}P < 0.05$; $^{**}P < 0.001$.

results provided by our system were compared to objective measures of visceral and cellular suffering. Lactate is the end product of the anaerobic energetic metabolism and an increase in lactate levels reflects hypoxic-ischemic conditions.

Systemic capillary lactate is of prognostic interest in mesenteric ischemia but its increase is slow and not suitable for early diagnosis.[20] Local capillary lactate,[21] directly sampled at the ischemic site and measured with the Lactate Pro device (LT170, Arkray, KGK, Japan), appeared more accurate in an ischemic leg model showing both rapid increase during ischemia and a rapid decrease during reperfusion. In our experimental study, we aimed to evaluate the capillary lactate level at the ROIs outlined by the AR using the Edge handheld device. The system provided a fast, consistent, and quantitative evaluation of the grading of ischemia in this 1-hour model of ischemia. The fluorescence-based laparoscopic augmented-reality system allowed to precisely identify the resection line separating the ischemic zone from vascular areas with a good correlation between fluorescence time-to-peak and capillary lactate level ($\rho = 0.71$ for the ischemic area).

Mitochondria are the main source of energy of cells through a process known as "oxidative phosphorylation." This is a complex phenomenon. Schematically, it starts with a series of redox reactions in which electrons are transferred from donors (NADH or QH_2) to the most electronegative acceptor, which is oxygen (O_2). This electron transport chain produces the energy necessary to generate an electrochemical gradient across the mitochondrial membrane by

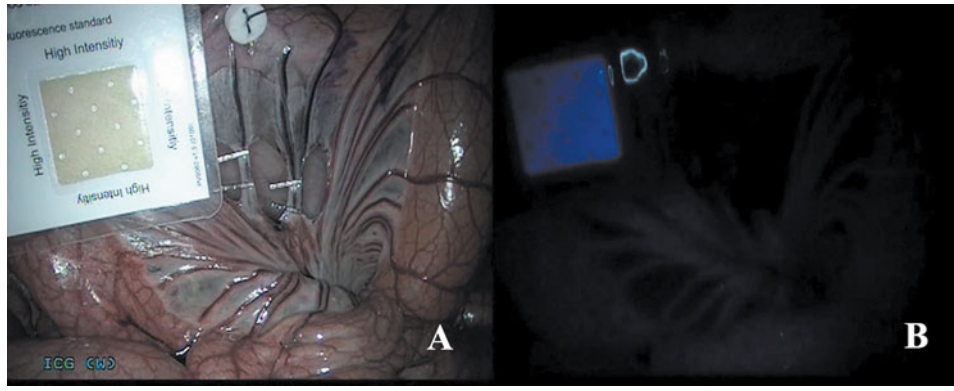

FIGURE 3. Laparoscopic view of the calibration aid. A calibration aid consisting in a sterile plastic card with a squared spot that gives a constant signal when illuminated by NIRS (Pulsion Medical) was inserted through a small laparotomy and used as a reference for fluorescence intensity to eliminate the variable "distance" between light source and target. (A) White light laparoscopic image showing the calibration aid close to the ischemic small bowel loop. (B) Near-infrared illuminated image showing fluorescence intensity of the reference spot as compared to fluorescent mesenteric vessels.

actively pumping H+ protons into the intermembrane space. The transmembrane gradient is used to produce the "cellular currency of energy," adenosine triphosphate. There are 3 enzymatic proton pumps: complexes I, III, and IV.

Ischemic lesions, by reducing the presence of O_2 as terminal electron acceptor, impair complexes I, III, and IV, and the transmembrane gradient is consequently abolished with energy depletion and cellular necrosis.[13] In our 1-hour model of mesenteric ischemia, we found an already significant impairment of respiratory chain in the ischemic zone as compared with the nonischemic segments as expressed by the V_{max} and V_{succi}. The fluorescence-based AR could effectively differentiate the small bowel zones presenting these very early signs of energetic suffering. The preserved activity of complex IV (V_{tmpd}) can be easily explained by the short duration of the ischemia and is a sign of cellular viability, because the reactions depending on complexes I and III are ready reversible in the ischemia-reperfusion models. It would be of particular interest to assess the mitochondrial respiration at different time points to exactly establish the point of no return and if fluorescence AR would be able to exactly determine whether this limit has been outreached.

There are 2 main techniques to analyze the metabonome: Mass spectroscopy and NMR spectroscopy. NMR spectroscopy is rapid, cost-effective, nonselective (i.e., it removes bias for a specific molecule), and by this sample preparation requires minimal manipulation and gives metabolic information on intact biological tissues. All in all, the principle of NMR spectroscopy is similar to the MR imaging process. The sample is placed into a magnetic field that aligns molecular nuclear spins. The application of a radiofrequency pulse enhances the energy level by inverting spin orientation, and the magnetization of the sample is deflected away from the magnetic field. Switching off the radiofrequency pulse allows the spins to relax to the original and stable energy level. This modification produces a detectable NMR signal that can be analyzed. Each molecule is identified by a series of spikes in the spectrum (Fig. 4) and the area under the peak is proportional to the concentration. The NMR spectrum of most metabolites has been analyzed and new spectra may be extrapolated on the basis of the available metabolic fingerprints. NMR spectroscopy has been used to characterize malignant tissues.[22,23] Our laboratory of membrane physics reported on the usefulness of metabonomic profiling to determine the adequacy of lung transplant.[24] Vincenti et al[25] assessed metabonomic profiling in small bowel samples after induction of intestinal ischemia-reperfusion injury by mesenteric artery or portal vein outflow occlusion in rats. Similarly to our study, the authors found dramatic changes in the intestinal biochemical profile and outlined 9 metabolites (out of 43 quantified) as being significantly different among the mesenteric artery, portal vein, and sham groups. Among these metabolites, there were glucose, lactate, and amino acids such as glutamate, glutamine, methionine, valine, leucine, and isoleucine.

In our model, metabolic profiles demonstrated decreased glucose levels and increased tissue lactate and amino acids following ischemia, showing an increased anaerobic glycolysis. The significant increase of choline, ethanolamine, sylloinositol, and myoinositol, which are phospholipids involved in the cellular membrane integrity, may represent an early sign of cellular ischemic damage. Interestingly, in our study, the glutathione, which is one of the most important endogenous antioxidant functions, and thereby an important marker of oxidative stress,[26] was only marginally modified and was not different in the ischemic, marginal, and vascular zones. This is due to the short duration of ischemia in our model (1 hour), which is not sufficient to generate irreversible tissue damage. The same consideration applies to glutamate, glutamine, and creatinine, which were also within normal value range. The small bowel samples analyzed for metabolic profile were taken after the vascular cartography generated by AR in a randomized fashion to reduce the influence of the time "ischemia start-to-sampling" of a specific ROI in the analysis, because metabolites are extremely labile and all metabolic activity has to be blocked immediately after sampling (by snap freezing tissue).

To summarize, there was a strong agreement between the fluorescence-based AR discrimination of the intestinal perfusion at the ROIs and different markers of cellular energetic status and metabolism: local lactates, mitochondrial respiration rate, and metabonomic profiling. The main aim of the present study was to show the feasibility of the technique and to identify precise outcomes to allow for the reduction in the number of experimental subjects. In fact, the outcome of anastomotic leak or stricture would require a very large number of animals to evaluate the superiority of fluorescence-based AR to determine intestinal viability when compared with clinical assessment alone. The surrogate use of cellular suffering markers (metabonomics and mitochondrial respiratory rate) might considerably reduce the number of experimental subjects. Further experimental studies to evaluate the clinical interest of this technology including survival models with digestive resections and anastomoses are currently underway at our institute.

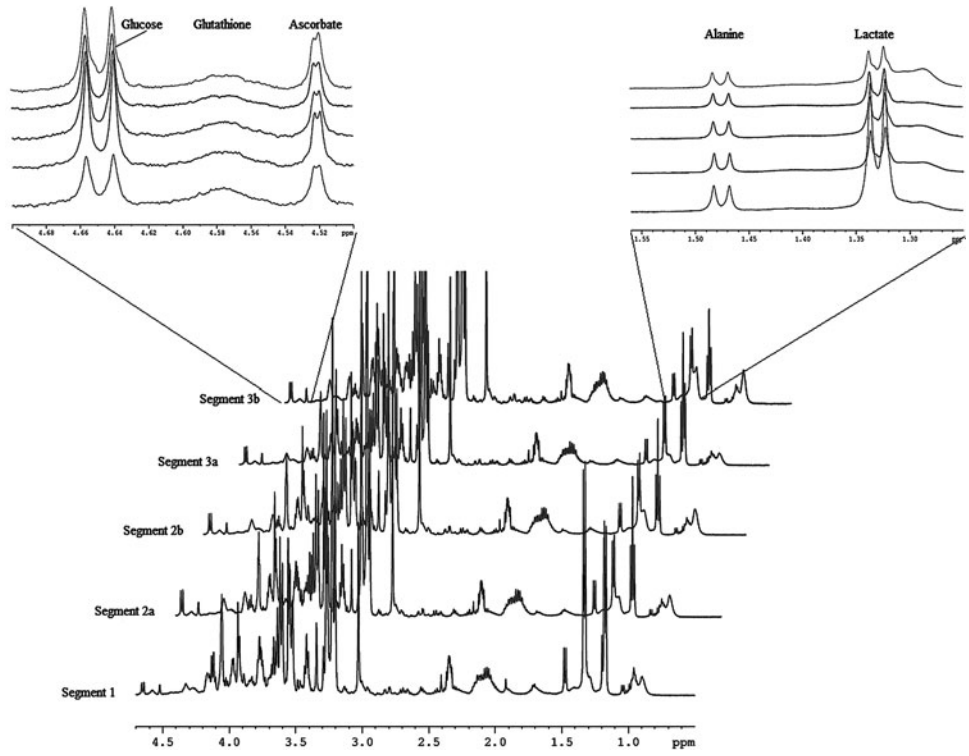

FIGURE 4. NMR high-resolution magic angle spin spectra. ^{1}H NMR HRMAS spectra performed on pig bowel biopsies obtained after AR identification of the ROI: ischemic zone (segment 1), marginal zones (segments 2a and 2b), and vascularized zones (segments 3a and 3b). Each molecule is identified by a series of spikes in the spectrum and the area under the peak is proportional to the concentration (nmol/mg of tissue).

CONCLUSIONS

This experimental study gives robust evidence that fluorescence-videography-based real-time AR system may effectively detect the boundary between the ischemic and the vascularized area in a laparoscopic model of mesenteric ischemia. Further experimental studies are underway to evaluate this technology to intraoperatively determine intestinal perfusion and viability at the future anastomotic site in various surgical procedures to reduce the risk of anastomotic or leak or stricture.

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