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**Detection of Thrombosis in Microvessels with Indocyanine Green Videoangiography**

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**Short Running head:** Intraoperative Detection of Thrombosis in Vascular Procedures

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**Abstract**

Atherosclerosis, a systemic condition responsible for multiple diseases and problematic in cases where plaque formation occurs at multiple sites. Thrombotic embolus formation may jeopardize vascular procedures including microvascular anastomoses in replantation surgery or free tissue transfer. A valuable pre- or intraoperative mobile imaging tool for detection of thrombosis would have advantages in this situation. An intimal injury, simulating removal of atherosclerotic plaque formation, was performed microsurgically in 60 rat aortas. Sequela were analyzed macroscopically, histologically and with intraoperative indocyanine green (ICG) videoangiography immediately postoperatively. The Spearman and Pearson correlation tests were used to compare the three modalities. Sensitivity and specificity of ICG videoangiography was calculated in relation to both macroscopic and histological results. Detection of thrombosis was possible in 25 cases and in 18 cases no thrombosis was correctly diagnosed by all methods used. In 31 of 60 specimens (51.7%), thrombus formation was detected histologically and in 29 of 60 examinations clinically (48.3%), yielding a correlation rate of 93.5% between both examinations. Macroscopic analysis showed a higher correlation with ICG videoangiography (sensitivity: 86.2% and specificity: 64.5%) than histological observations compared with ICG videoangiography (sensitivity: 80.6% and specificity: 62.1%. Between all modalities a significant correlation was detected (each $p \leq 0.001$) with a correlation index of 0.94, 0.52 and 0.44 for macroscopic /histological, clinical/ICG videoangiographic, and ICG videoangiographic/histological results, respectively. We show that ICG videoangiography adds an important method to detect acute thrombus formation and may be an important tool in vascular procedures.

**Key words:** ICG videoangiography; thrombus prediction; endothelial defects

## Introduction

Microvascular surgery has become a routine operative procedure in reconstructive surgery [1]. Despite its universal integration into clinical practice there remain some limitations and drawbacks to this procedure. Systemic vascular disease, which can affect both the donor and recipient vessels in microvascular tissue transfers [2, 3] is often regarded as a relative contraindication due to the presence of atherosclerosis. Typically, plaque is observed as a focal intimal deformation with the accumulation of lipids, carbohydrates, blood products, fibrous tissue, and calcium [4]. These lesions increase in size and tend to spread with age. Atherosclerotic plaques need to be factored into the choice of techniques, planning, and expected complications [2, 3, 5]. Patient selection, type of microsurgical technique, choice of an adequate flap, and postoperative care are also very important in these patients [2]. Injuries of the arterial vessel wall have been shown to be associated with an increased risk of thrombus formation in cases of vertical lesions, raising the possibility of plaque endarterectomy in microvessels without an increased thromboembolic risk in selected cases [6].

The injected ICG dye binds strongly to plasma globulins, remains intravascular and was used in several studies as a fluorescence marker for intraoperative assessment of arterial and venous perfusion [7, 8]. This technique was also applied for the immediate analysis of patency following microvascular anastomosis as an integrated system in the operating microscope and showed promise [9, 10]. A further development is the FLOW® 800 tool which is a integrated software of the operating microscope (OPMI® Pentero®; Carl Zeiss Meditec AG; Oberkochen, Germany) with a near-infrared videoangiography detection system (INFRARED 800; Carl Zeiss Meditec AG; Oberkochen, Germany). This allows immediate quantitative flow measurements based on intraoperative ICG videoangiography [11].

The aim of this study was to compare three different diagnostic instruments for the detection of adherent thrombosis after intimal injury in a rat aorta model, simulating removal of atherosclerotic plaque formation. We compare the macroscopic and histological assessment of thrombus formation (the gold standard) with intraoperative ICG videoangiography.

## Material and methods

### Ethical statement

All animals were cared and housed in accordance with the EU-guidelines. The study was approved by the regional government (Regierung von Oberbayern, AZ 55.2-1-54-2532-3-35-08) and was conducted in accordance with the German Animal Welfare Act. A total of 60 male Wistar rats (280–320g, Fa. Charles River, Kißlegg, Germany) were used. Food and water were provided freely. All surgical procedures were performed under aseptic conditions and intravenous general anesthesia [ketamine 100mg/kg (Narketan®, Fa. Vétoquinol GmbH, Ravensburg, Germany) and xylazine 5 mg/kg (Rompun®, Fa. Bayer Vital GmbH, Leverkusen, Germany)] using the femoral vein access as previously decribed [12].

### Surgical Technique

After induction of anesthesia, the animals were placed on a work pad in supine position and a ventral, median abdominal incision of 4 cm length was performed. Following the abdominal aorta between the renal arteries and the aortic bifurcation was freed from perivascular tissue and all aortic branches in this section were ligated and cut. As described in detail previously, the infrarenal aorta was temporarily clipped proximally and distally followed by longitudinal incision of 10 mm length [6]. After standard preparation of the exposed lumen including rinsing with physiological saline solution, endothelial defects of varying sizes were performed by removing the endothelium surgically under the operating microscope on the opposite site of the longitudinal incision. Here a perforation of the vessel wall was prevented by meticulous preparation. Following endothelial defect preparation, the lumen was rinsed, the infrarenal longitudinal incision was closed with interrupted sutures using 11-0 Ethilon

(Ethilon®; Ethicon Division of Johnson & Johnson; Livingston, Scotland) and the temporary clips were removed for re-establishment of blood flow for one hour under continued anesthesia for final ICG videoangiography.

## ICG Videoangiography

Blood flow after one hour under continued anesthesia was assessed using the OPMI® Pentero® integrated near-infrared videoangiography detection system with the FLOW® 800 tool (INFRARED 800; Carl Zeiss Meditec AG; Oberkochen, Germany) [9, 13]. As previously decribed in detail, the ICG dye (ICG-PULSION; Pulsion Medical System AG; Munich, Germany) was injected weight-adapted intravenously (0.3 mg/kg body weight, 25 mg dissolved in 5 ml sterile water) as a bolus into the femoral vein using a microcatheter (Premicath; VYGON GmbH & Co. KG; Aachen, Germany) [14]. The ICG videoangiography started immediately after injection and was recorded in real time over a period of 120 seconds at a fixed working distance of 300 mm and with a 15-fold magnification. All data were immediately analyzed and stored. The detection and FLOW® 800 analyses using a integrated mathematical softwaretool (FLOW® 800; Carl Zeiss AG; Oberkochen, Germany) is described earlier in detail [14, 15]. The resulting fluorescence intensity was recorded as arbitrary units (AU) and both color encoded figures as well as angiographies were analyzed by two independent, blinded investigators for the detection of thrombus formation (TM and CW).

## Postoperative Analyses

Immediately after ICG videoangiography, the rats were euthanized while still in deep anethesia by using a combination of lethal pentobarbital injection (200 mg/kg,

Narcoren®, Rhone-Merieux, Laupheim, Germany) and exsanguinating following suprarenal of the abdominal aorta. All aortas were macroscopically inspected and the visual incidence of thrombus formation was noticed.

Following, the excised part of the abdominal aorta was prepared for further histological analyses with the main focus on the incidence of thrombus formation using a standardized and previously reported protocol [16]. Areas 500 μm proximal and distal to the longitudinal incision were specifically analyzed. All histological observations were independently evaluated by two blinded investigators to the macroscopic and ICG videoangiogaphic results (AMF and LMR). The results were documented with an integrated CCD camera (CAMEDIA C5050; Olympus; Hamburg, Germany) on a Zeiss Axioskop with magnification lenses 1.25, 10, 20, 40 and 63 oil.

Statistics

The SPSS software package (SPSS 23, SPSS Inc.; Chicago, IL, USA) was used for statistical analysis. The Spearman and Pearson correlation test was used to compare clinical observation, histological analysis, and ICG videoangiography with each other. Sensitivity and specificity of ICG videoangiography was calculated in relation to both clinical and histological results. Differences were considered statistically significant for a two-sided exact p value of $< 0.05$. All data are presented as mean ± standard deviation (±SD).

## Results

All 60 rats survived the operation with no perioperative issues. The operation lasted a mean time of 75 ± 12 minutes. The mean defect size was 2.7 ± 1.2 $mm^2$ [range 0.8 to 5.4 $mm^2$]. In 31 of 60 specimens (51.7%), thrombus formation was detected histologically and in 29 of 60 examinations macroscopically (48.3%), yielding a correlation rate of 93.5% between both goldstandard examinations. In 36 of 60 specimen (60%) thrombus formation was detected by ICG videoangiography.

### Robustness of methods

Detection of thrombosis was possible by all methods in 25 cases. In another 18 cases no thrombosis was correctly diagnosed by all methods.

Comparisons of both macroscopic and histological examinations (Fig. 1) in comparison with ICG videoangiographic results are presented in Table 1. Macroscopic observations showed a higher correlation with ICG videoangiography, yielding a sensitivity of 86.2% and specificity of 64.5%. Histological observations compared with ICG videoangiography showed a sensitivity of 80.6% and specificity of 62.1% (Table 2, Fig. 2 and 3).

### Spearman and Pearson correlation

Between macroscopic and histological results a significant correlation was detected ($p<0.001$) with a high correlation index of $r = 0.94$. Comparing the macroscopic and ICG videoangiographic results, a significant correlation was also detected ($p<0.001$) with a correlation index of $r= 0.52$. Histological results revealed also a significant correlation in comparison with ICG videoangiographic results ($p=0.001$) with a

correlation index of r= 0.44. This indicates, that all methods investigated showed consistent results with a high correlation and conformity.

## Discussion

The purpose of many studies regarding arteriosclerosis detection and micro- or macrovascular surgery is to describe a method that predicts a thrombus sufficiently.

Our presented study shows the value of ICG videoangiography in the detection of thrombus formation in microvessels simulated by endothelial lesions in the rat [6]. This is, to the best of our knowledge, the first study investigating the detection of thrombus formation in a microvascular experimental model with reference to histological and macroscopic visual assessment allowing correlation between the applied methods. Based on the current findings, we would suggest that ICG videoangiography is a valid and robust tool in the detection of acute thrombus development.

Detection of thrombus formation is still important in micro- and macrovascular vessels. Atherosclerosis is an important risk factor for its occurrence and acute development after surgical procedures [2, 17]. The general condition of patients with a history of atherosclerosis is routinely assessed preoperatively but risks of non-detection still remain. Widely available techniques such as computed tomography angiograms, magnetic resonance imaging and others can be performed but a risk of false negative or positive results remains [18-20].

Only a few experimental studies exist evaluating the sensitivity and specificity of diagnostic methods in comparison to histological or macroscopic findings. In an experimental animal study using a porcine coronary model by Maeng et al., angioscopy detected thrombus formation with a sensitivity of 90% and a specificity of 50% [21]. They conducted 39 experiments, less than us. The corresponding positive and negative predictive values were 65% and 63%, respectively [21]. A study by Siegel et al. shows similar results for angioscopy and ultrasound for the detection of thrombus formation in coronary arteries [22]. Comparing these results to our data, our method has a high robustness with more valid results. In addition our method is a comparable invasive

procedure to angioscopy, but with only rare side-effects of the ICG dye compared to the commonly needed contrast agents [23]. Furthermore, the ICG dye is easily injected in a peripher venous catheter. Therefore it is a promising tool for introduction into clinical practice.

Zhao et al. investigated detection of thromboembolic event in an atherosclerotic rabbit model, induced by balloon injury within the abdominal aorta [24]. After additional de-endothelialization of the aorta and pharmacological triggering PET/CT was conducted for the detection of thrombus formation [24]. After gross anatomical analysis and histopathological evaluation similar to our study, 15 of 23 thrombi were correctly diagnosed. After adaption of the $^{18}$F-FDG uptake based on the morphological findings a maximal sensitivity of 75.4% and specificity of 88.5% with a positive and negative predictive value of 53.5% and 98.5% was achieved [24]. In contrast to this method ICG videoangiography has no radiation and is easily performed in the intraoperative setting, since its function is based on simple fluorescence angiography.

The advantages of ICG videoangiography compared to the other mentioned methods are cost-effectiveness and radiation free analyses. Therefore the clinical application compared to the others is feasible and furthermore associated with compareable results.

Limitations

One limitation of the present study is that our results are based on a simulated rat model not necessarily comparable to clinical situations such as stable arteriosclerotic plaque formation. In addition, the thrombi observed were mainly protrusive or mural thrombi, although occlusive thrombi were also detected. The course of each thrombus cannot be predicted. Therefore our findings need to be interpreted with caution since the values might be different in humans and furthermore fluctuate between patients

due to disturbances in hemodynamics, application of vasoactive substances or physiological variations in microcirculation [25].

## Conclusions

In conclusion, detection of thrombus formation in our rat simulation model was associated with a high sensitivity and moderate specificity with moderate-to-high predictive values compared with a very highly sensitive and specific histological method. We found a significant association between the three different diagnostic methods used in this experimental study. We believe that ICG videoangiography adds an important, non-invasive method to detect acute thrombus formation and may have an important place in future interventional vascular macro- and microvascular surgery as well as in the field of interventional radiology.

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## Conflict of interest

The authors declare no conflicts of interests.

## Disclosure

The authors report no proprietary or commercial interest in any product mentioned or concept discussed in this article.

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## Tables

**Table 1.** Results and conformity to detect thrombus formation of macroscopic and histological results in comparison with ICG videoangiography.

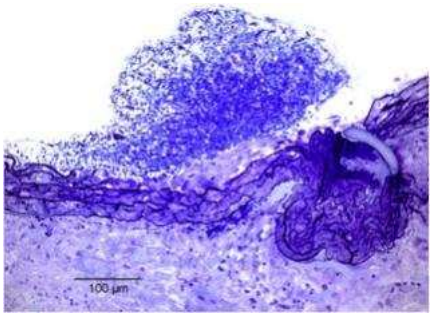
**Table 2.** Robustness of ICG videoangiography in comparison to macroscopic and histological detection of thrombus formation.

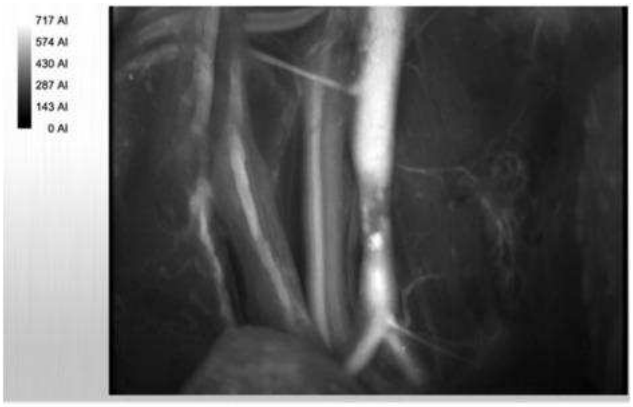
**Figure legend**

**Figure 1.** Histological diagnosis of an acute and growing thrombus arising from an endothelial defect.

**Figure 2.** Detection of an acute and growing thrombus formation by ICG videoangiography (above) and evaluation by FLOW® 800 (bottom). The thrombus formation is already established at the vessel wall indicated by lower enhancement of the fluorescence in the center. It was still growing, which is shown by the higher fluorescence signal at the bottom of the lesion. The FLOW 800® figure (bottom) indicates the growing part by color encryption.

**Figure 3.** Example of an acute thrombus formation indicated by fluorescence enhancement in the aortic lesion. The FLOW® 800 (bottom) indicates the already stable thrombus formation, whereas the native ICG videoangiography indicated the growth of thrombus formation (above).


100 µm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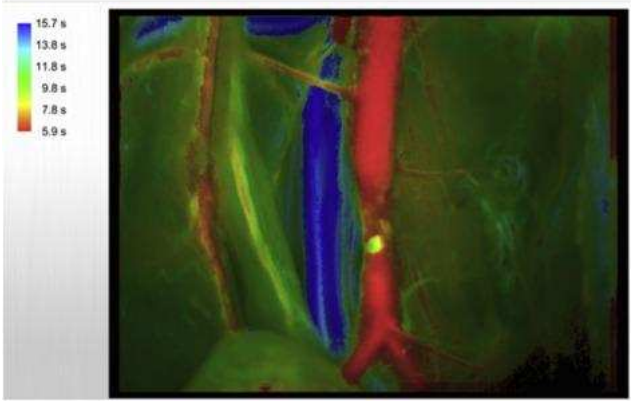
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13.8 s
11.8 s
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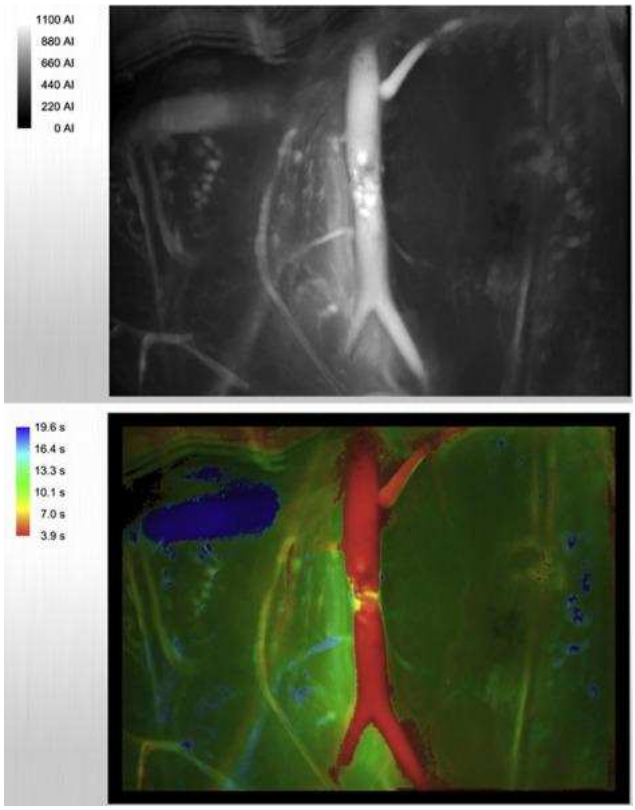
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440 AI
220 AI
0 AI
19.6 s
16.4 s
13.3 s
10.1 s
7.0 s
3.9 s