4-Experimental Surgery

# Fluorescent test accurately predicts leak of ischemic colon anastomosis in rats[1]

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**Abstract**

**Purpose:** To investigate if fluorescein fluorescent test can predict dehiscence in a model of ischemic colonic anastomosis in rats.
**Methods:** This experimental controlled trial randomly assigned 55 rats to four groups. Anastomoses were performed in non-ischemic colon segments (control group) and in ischemic colon segments measuring 1, 2 or 3 cm long (groups 1, 2 and 3, respectively). Fluorescein was injected and the tissues were examined under ultraviolet light. Seven days later, a second-look surgery was performed to check for the presence or absence of anastomosis dehiscence.
**Results:** Twenty-four rats presented anastomotic dehiscence during the second-look surgery. Reticular and nonfluorescent patterns were significantly associated with the occurrence of anastomotic dehiscence. Fluorescein fluorescence had a sensitivity of 95.8%, specificity of 89.2%, positive predictive value of 88.4%, negative predictive value of 96.2%, and accuracy of 92.3% to predict anastomotic dehiscence.
**Conclusion:** Fluorescein fluorescent test can accurately predict leak in a model of ischemic colonic anastomosis in rats.

**Key words:** Anastomosis, Surgical. Anastomotic Leak. Fluorescence. Blood Supply. Rats.

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## ■ Introduction

Surgeries that require anastomosis of the large bowel are among the most commonly performed abdominal procedures in the western world[1]. Ongoing advances in terms of perioperative care and surgical techniques have provided better results in the surgical treatment of patients with colorectal diseases. However, the rate of complications after colorectal surgeries is still high - around 30% -, and anastomotic dehiscence remains a relevant unsolved problem[2-5].

There is a general consensus on the key role of blood supply, mainly at the bowel margins, for the proper healing of intestinal anastomosis. Therefore, a rigorous intraoperative evaluation of the intestinal perfusion is essential for the prevention of anastomotic dehiscence[1,6]. Clinical tests to predict the occurrence of anastomotic dehiscence have very low accuracy, with a failure rate up to 60%[4,7-9]. Several studies have been conducted to find an objective and reliable method of intraoperative evaluation of intestinal blood supply in the last four decades[2,3,7,10-14]. To present date, no method has been accepted as the standard for this matter, however fluorescence tests have gained some attention[16-22].

Fluorescein fluorescence test is done by means of injection of intravenous sodium fluorescein, followed by examination of target tissues under ultraviolet light in a darkened room. Luminescence clearly differentiates well perfused from ischemic tissues[12]. The sensitivity and specificity of this technique are close to 100%[11,14]. Currently, fluorescence method to evaluate the perfusion of intestinal anastomoses has been increasingly used in robotic-assisted and laparoscopic colorectal resections, such as left colectomies and rectal ressections.

The aim of this study was to determine the sensitivity, specificity, positive predictive value (PPV) and negative predictive value (NPV) of fluorescein fluorescence test to predict anastomotic dehiscence.

## ■ Methods

This study was approved by the Research Ethics Committee and the Ethics Committee for the Use of Animals (Protocol 110668) of the Hospital de Clínicas de Porto Alegre. This study has been carried out in accordance with The Code of Ethics of the World Medical Association (Declaration of Helsinki).

Surgical procedures were conducted in the Animal Experimentation Unit of Hospital de Clínicas de Porto Alegre (AEU-HCPA). Male Wistar rats, 10 to 12 weeks old, from contemporary litters (275-340g) were purchased from the Vivarium of the Federal University of Pelotas (Pelotas, Brazil). Animals were maintained in groups of four in separate cages in a temperature-controlled room (22 ± 2ºC) and 12 hour light-dark cycle. All the animals received free access of water and standard rat chow. The rats were daily monitored by veterinarians from the AEU-HCPA.

This experimental controlled trial randomly assigned rats to four groups. The sample size of each group was calculated based on the results of a pilot study conducted with 20 rats to investigate the rate of anastomotic dehiscence according to the different lengths of intestinal devascularization. With regard to the expected frequency of 45% of overall anastomosis dehiscence in that study, a sample size of 55 rats was considered sufficient to achieve a sensitivity of 99% and a specificity of 91%, at 5% precision for sensitivity and 10% for specificity and a level of confidence of 95%. The minimum number of rats required for statistical analysis was used in each of the following groups: control group (nonischemic colocolonic anastomosis), and groups 1, 2 and 3 (colocolonic anastomosis in ischemic segments of 1, 2 and 3 cm-long, respectively) (Figure 1).

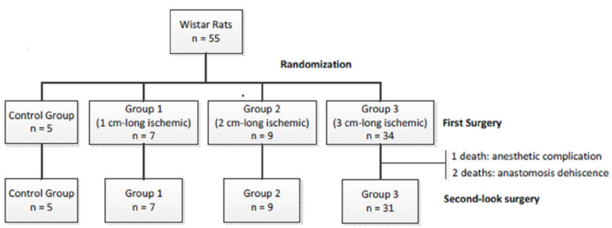


**Figure 1** - Study design and randomization of groups.

*Anesthesia and surgical procedure*

The rats were weighed and given intraperitoneal injection of ketamine (90 mg/kg) and xylazine (10 mg/kg). After loss of paw withdrawal reflex was confirmed, the animals were fixed to a temperature-controlled operating table and had the abdomen region shaved and aseptically cleaned with 10% topical povidone-iodine. Sterilized surgical instruments were used for the surgical procedures[23]. All animals underwent midline laparotomy, abdominal exploration and exposure of ascending colon. The control group underwent transversal section of the ascending colon. A straight colocolonic anastomosis was performed with a single layer of continuous suture with 6-0 polypropylene monofilament. In groups 1, 2 and 3, a colon segment of 1-, 2- and 3-cm long, respectively, underwent selective devascularization, as previously described by Griffen24. This method consists of devascularization of the marginal artery at two points, as well as the vasa recti located in-between, using a bipolar cautery[1]. At the midpoint of the devascularized segment, the colon was sectioned and an anastomosis was performed in the same way as described in control group. None of the anastomoses showed clinical signs suggestive of an immediate anastomotic ischemia.

*Fluorescein fluorescence test*

All animals underwent fluorescein fluorescence test. Fluorescein fluorescence test is done by injecting fluorescein sodium (1.5 mg/100 mg diluted in saline solution at 3 mg/mL) intravenously, followed by examination of target tissues under ultraviolet light (16 watts Wood lamp, Ramsor®, São Paulo, Brazil) in a darkened room. The test was conducted immediately after the performance of the anastomosis. The 2-cm colon segments in each side of the anastomosis were evaluated to access the fluorescence pattern using a +4-diopter lens. The fluorescence patterns were classified as homogeneous, reticular, patchy or nonfluorescent[12,14] (Figure 2).

The abdominal wall was then closed

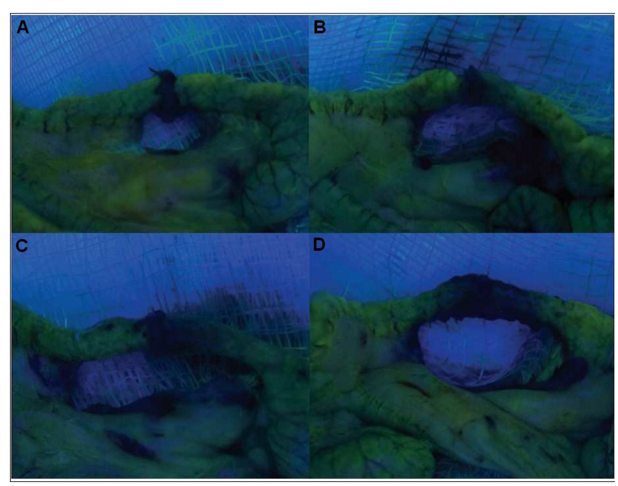


**Figure 2** - Fluorescein fluorescence patterns: **A)** homogeneous; **B)** reticular; **C)** patchy; **D)** nonfluorescent.

with two layers of continuous suture with 4-0 nylon and thereafter an intraperitoneal injection of tramadol hydrochloride (5 mg/kg) was administered for postoperative analgesia. Seven days later, the rats underwent a second-look surgery for macroscopic re-evaluation of the anastomosis and reassessment of the fluorescein fluorescence test. Surgical findings were classified according to the following degrees: grade 1 - ascending colon with normal appearance or minor edema; grade 2 - marked edema or signs of ischemia (purplish color, darkened areas); grade 3 - partially blocked dehiscence or abscess around anastomosis; grade 4 - totally blocked dehiscence or peritonitis due to total or partial dehiscence. Subsequently, the animals were killed by exsanguination.

*Statistical analysis*

The results of the descriptive analyses for continuous variables were expressed as means and standard deviations (SD). The results for qualitative variables were reported as absolute frequencies and proportions. Chi-square test was used to analyze comparisons between groups. Mann-Whitney and Kruskal-Wallis tests were used to analyze asymmetrically distributed continuous variables. The level of significance was set at 5%.

## ■ Results

A total of 55 rats were used in this study: five rats in control group, seven rats in group 1, nine rats in group 2, and 34 rats in group 3. Table 1 presents general data for each group. Rats in group 3 were significantly younger than those in the other groups (78 vs. 78.7 vs. 78.3 vs. 74.2-days-old; $p < 0.001$). There were no statistically significant differences between the groups in terms of baseline and re-intervention weights and surgery duration.

**Table 1** - General data for each intervention group.

| | Control Group mean (25-75$^{th}$) | Group 1 mean (25-75$^{th}$) | Group 2 mean (25-75$^{th}$) | Group 3 mean (25-75$^{th}$) |
|---|---|---|---|---|
| Age (days) | 78 (77–79.5) | 78.7 (77-80) | 78.3 (77-79.5) | 74.2 (71.3-79)* |
| Baseline weight (g) | 309.2 (282-335) | 322.3 (286-368) | 333.5 (305-364.5) | 316.6 (291.5-332.5) |
| Surgery duration (min) | 24 (22-26.5) | 22.4 (20-24) | 25.9 (22.5-28.5) | 24.8 (23-26.3) |
| Re-intervention weight (g) | 291.4 (269.5-314) | 293 (242-340) | 311.7 (283.5-345) | 293.8 (268-322.3) |
| Re-intervention duration (min) | 10.2 (9-11) | 9.9 (8-11) | 10.7 (9-11) | 10.6 (9-12) |

*$p<0.001$

Three rats died in group 3; one as consequence of anesthetic complication after the first surgery and two due sepsis secondary to anastomotic dehiscence, confirmed at the necropsy. These rats were excluded from the statistical analysis. The fluorescein fluorescence patterns in the first surgery, as well as the surgical findings and fluorescein fluorescence patterns in the second-look surgery are demonstrated in Table 2. All rats in the control group had homogenous fluorescence pattern in both surgeries and normal appearance or minor edema (grade 1) in the second-look surgery. All rats in group 1, except one (reticular pattern), presented homogeneous fluorescence pattern and normal appearance or minor edema of colon (grade 1). This sole rat with reticular pattern experienced anastomotic dehiscence and was classified as grade 3 (partially blocked dehiscence or abscess around anastomosis) during the second-look surgery. The majority (77.8%) of the rats in group 2 presented reticular fluorescence pattern in the first surgery ($p < 0.01$). Most of these rats (85.7%) maintained the same fluorescence pattern during the second-look surgery ($p < 0.01$) and none experienced anastomotic dehiscence.

Rats in group 3 presented mostly patchy or nonfluorescent patterns both in the first and the second-look surgery, respectively (55.9% and 29.4%, $p < 0.05$; 58.1% and 25.8%, $p < 0.01$). Twenty-three (74.2%) rats in group 3 presented partially blocked dehiscence or abscess around anastomosis (grade 3) during the second-look-surgery ($p < 0.01$).

**Table 2** - Fluorescein fluorescence pattern and surgical results for each intervention group.

| | Control Group n (%) | Group 1 n (%) | Group 2 n (%) | Group 3 n (%) |
|---|---|---|---|---|
| First surgery fluorescein fluorescence pattern (n = 55) | | | | |
| Homogeneous | 5 (100)* | 6 (85.7)* | 2 (22.2) | 0 (0) |
| Reticular | 0 (0) | 1 (14.3) | 7 (77.8)* | 5 (14.7) |
| Patchy | 0 (0) | 0 (0) | 0 (0) | 19 (55.9)* |
| Nonfluorescent | 0 (0) | 0 (0) | 0 (0) | 10 (29.4)* |
| Second-look surgery fluorescein fluorescence pattern (n = 52) | | | | |
| Homogeneous | 5 (100)* | 6 (85.7)* | 3 (33.3) | 0 (0) |
| Reticular | 0 (0) | 1 (14.3) | 6 (67.7)* | 5 (16.1) |
| Patchy | 0 (0) | 0 (0) | 0 (0) | 18 (58.1)* |
| Nonfluorescent | 0 (0) | 0 (0) | 0 (0) | 8 (25.8)* |
| Second-look surgical findings (n = 52) | | | | |
| Grade 1 | 5 (100)* | 6 (85.7)* | 4 (44.4) | 0 (0) |
| Grade 2 | 0 (0) | 0 (0) | 5 (55.6)* | 8 (25.8) |
| Grade 3 | 0 (0) | 1 (14.3) | 0 (0) | 23 (74.2)* |
| Grade 4 | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Anastomosis dehiscence | 0 (0) | 1 (14.3) | 0 (0) | 23 (74.2) |

*p<0.001

As shown in Table 3, the homogeneous and reticular fluorescence patterns immediately after the anastomosis construction were significantly associated with anastomotic integrity during the second-look surgery. In contrast, the reticular and nonfluorescent patterns were significantly associated with the occurrence of anastomotic dehiscence.

**Table 3** - Incidence of anastomotic dehiscence according to fluorescence pattern in the first surgery.

| | Homogeneous n (%) | Reticular n (%) | Patchy n (%) | Nonfluorescent n (%) |
|---|---|---|---|---|
| No dehiscence | 13 (46.4)* | 12 (42.9)* | 3 (10.7) | 0 (0) |
| Dehiscence | 0 (0) | 1 (4.2) | 14 (58.3)* | 9 (37.5)* |

*p<0.001

Due to the similarity of data and same clinical meaning, the results of homogeneous and reticular patterns, as well as those of patchy and nonfluorescent patterns, were combined for calculations of sensitivity, specificity, positive and negative predictive values and accuracy of fluorescein fluorescence for anastomosis dehiscence. Data are shown in Table 4.

**Table 4** - Diagnostic accuracy of fluorescein fluorescence test for anastomotic dehiscence.

| | Result (%) | 95% CI |
|---|---|---|
| Sensitivity | 95.8 | 76.9 – 99.8 |
| Specificity | 89.2 | 70.6 – 97.2 |
| Positive Predictive Value | 88.4 | 68.7 – 96.8 |
| Negative Predictive Value | 96.2 | 78.4 – 99.8 |
| Accuracy | 92.3 | 83.1 – 100 |

## ■ Discussion

Anastomotic dehiscence is the “Achilles heel” for colorectal surgery, despite the many technical advances achieved in recent decades. This can be explained by its high incidence and negative impact on the health of the patient (increased morbidity and mortality rates, length of hospital stay, risk of permanent ostoma, poor prognosis in cancer patients, damage on quality of life) and on the socio-economic status of the population (increased health costs and workplace absence)[25]. So far, there is no technique or cost-effective method for predicting the occurrence of colorectal anastomotic dehiscence[2,3,26]. Clinical parameters, such as intestine color, the presence of peristalsis, mesenteric pulse, active bleeding have historically demonstrated low accuracy to assess the viability of ischemic bowel[12]. More sophisticated evaluation methods of intestinal perfusion, such as laser Doppler fluxometry, intramural pH, intestinal electromyography, intra-arterial injection of dye and radioactive microspheres, have also been tested[12]. However, these techniques are invasive, expensive or require specialized equipment[12]. The ideal evaluation method of intestinal perfusion should be easily accessed in the operating room, quick to use, have good accuracy, low cost and be minimally-invasive[12]. The fluorescence method with intravenous administration of fluorescein and examination under ultraviolet light meet all these criteria[7,10-12,14].

The present study comprised a control group (non-ischemic anastomosis) and three intervention groups with varying degrees of ischemic colonic anastomosis in rats. The objective was to analyze the immediate and delayed patterns of fluorescein fluorescence in each group and to associate these findings with the occurrence of ischemic damage to the large intestine. We described, for the first time in the literature, an accurate method for the evaluation of perfusion of the ischemic colon segments in an experimental model to predict anastomosis dehiscence in rats. This pilot study showed that 3 cm long segment of the ischemic colon resulted in higher rates of anastomotic dehiscence. Following the recommendations of the Ethics Committee for the Use of Animals, group 3 had a greater number of rats than the others to ensure that the objective of this study was achieved with reduced number of deaths. The rats in group 3 were significantly younger compared to the other groups. No significant differences were observed between the average weight of the animals, which is an indirect indicator of nutritional status, a factor involved in the occurrence of anastomotic dehiscence in rats[27]. However, the highest rate of anastomotic dehiscence in group 3 could not be attributed to the fact that these rats were younger compared to those in the other groups. On the contrary, this finding can be attributed to the greater extent of colonic ischemia resulting from the intervention in group 3, as objectively described using the fluorescein fluorescence method. In fact, more than 80% of the rats in group 3 showed signs of critical tissue ischemia (patchy or non fluorescent pattern) both in immediate and late evaluation of anastomosis perfusion. These fluorescence patterns represent the total and regional disruption of blood flow of the colon, respectively. Both patterns were statistically associated with the finding of ischemic tissue damage in second-look surgery, particularly the anastomotic dehiscence. The analysis of the fluorescence test results showed high levels of sensitivity, specificity, PPV, NPV and accuracy of fluorescein fluorescence for anastomotic dehiscence.

This performance makes the fluorescein fluorescence test an appropriate method to indicate correctly the revision and re-confection of an ischemic colonic anastomosis, therefore, decreasing the rate of anastomotic dehiscence. It also allows the surgeon to accurately judge the adequacy of the perfusion of a colonic anastomosis, avoiding unnecessary revision and re-confection. It should be reminded that these results were observed in colonic anastomosis without clinical evidence of ischemic suffering immediately after its confection and should not be extrapolated to cases of clinically detectable ischemia.

In the last decades, several authors have investigated the use of fluorescence techniques to evaluate the feasibility of anastomosis in the left colon and rectum in both conventional and minimally-invasive surgeries (laparoscopic and robotic)[9,28]. These publications corroborate our findings that the fluorescence technique is able to accurately identify colonic anastomosis with critical ischemia, which requires modifications of surgical procedures (e.g. confection of a temporary deviation ostoma or, more often, an extension of the bowel resection and confection of a new anastomosis) to reduce the rate of anastomotic dehiscence[9]. Unlike the intraoperative near infrared fluorescence adopted in minimally-invasive surgery, especially the imaging platform - named Firefly system - integrated into the da Vinci robot[28], the fluorescein fluorescence technique under ultraviolet light is extremely cheap, making it an affordable diagnostic test to be used in developing countries.

The study has some limitations. First, we did not perform a histopathological study of the anastomosis for comparative analysis with the fluorescence test findings. Second, no other evaluation method of intestinal perfusion was used for comparison with the fluorescence test. Finally, there was no study on the eventual outcomes of the possible changes in surgical procedures, resulting from the interpretation of the fluorescence test findings. Even with these limitations, our study validated an easy to use, low cost and highly accurate method to predict ischemic colonic anastomosis in rats. Further studies are required to confirm whether these findings may be reproduced and are beneficial for humans.

## ■ Conclusions

Fluorescein fluorescent test accurately predicts leaks in a model of ischemic colonic anastomosis in rats. Notably, the reticular and nonfluorescent patterns are associated with the occurrence of anastomotic dehiscence

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