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Impaired Blood Supply in the Colonic Anastomosis in Mice Compromises Healing

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Colon anastomotic leakage has a multifactorial etiology and ischemia is considered one of the most important single factors. However, no existing animal models have established a direct link between ischemia and anastomotic leakage. The aim of this study was to establish a model of colon anastomotic leakage as a result of tissue ischemia. In colon anastomoses of 53 C57BL/6 mice, varying degrees of ischemia were induced. Supplying vessels were divided with bipolar coagulation in order to reduce anastomotic breaking strength and create clinical anastomotic leakage. Breaking strength of all the ischemic anastomoses were significantly lower compared with controls. Increasing ischemia resulted in higher rates of large bowel obstruction without creating anastomotic leakage. Healing was compromised as a result of impaired blood supply. However, clinical leakage was absent. Pure ischemia in otherwise healthy experimental animals may be too simple of an approach to create clinical leakage.

Key words: Colon anastomotic leakage – Animal model – Ischemia – Clinical anastomotic leakage

Anastomotic leakage remains a serious and frequent complication in colonic surgery. Smoking, coronary heart disease, and hypertension are some of the known risk factors for anastomotic leakage in the colon. Patients with such conditions have been shown to have microvascular disease in the anastomotic tissue, which may impair perfusion, creating ischemia and anastomotic leakage.[1–3]

Existing animal models inducing isolated colon anastomotic ischemia have failed to create clinical leakage with abscess or fecal peritonitis.[4,5] Using a combination of ischemia and technical insufficiency, other models have been able to create clinical leakage, but the effect of ischemia on the colonic anastomosis alone remains unknown.[6,7] Several animal studies have evaluated healing of the anastomosis without producing clinical leakage.[8] However, since clinical leakage is a condition comparable to the human scenario, a model for this may be needed. A validated model of the ischemic

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anastomosis could test new interventions aiming to reduce anastomotic leakage in high risk patients. The mouse is a well-suited animal for this purpose, since manifestation and cause of anastomotic leakage are comparable to the human condition.[8,9]

The purpose of this study was to create a new experimental model of clinical colon anastomotic leakage as a result of compromised blood supply in the mouse.

Materials and Methods

This study used 53 male C57BL/6 mice (23–30 grams), in which end-to-end colonic anastomoses were created.

Anesthesia and operation

The animals were anaesthetized with isoflurane (isoflurane 2%, O_2 1000 mL/minute). Initially, the abdomen was shaved and excess hair was removed with a piece of tape, after which the abdomen was disinfected. Midline incisions (1.5–2.0 cm) of both the skin and muscle layer were performed, through which the cecum was identified and pulled forward with 2 sterile swabs, exposing only the part of the bowel, which was used for the anastomosis. The colon and a small part of the mesentery 1 cm distal to the cecum were divided by micro scissors without damaging the vessels. Hereafter, an end-to-end colocolic anastomosis was constructed using 8 extramucosal, interrupted, and equidistant coated sutures (Vicryl 8-0; Ethicon, Inc., Cincinnati, Ohio) with a taper-point needle. The abdominal wall was closed using a 4-0 running suture (Vicryl 4-0, Ethicon, Inc.) in the muscle layer, and three interrupted sutures (Vicryl 4-0, Ethicon, Inc.) with inverted knots in the skin. Each animal received 0.2 ml saline subcutaneously as fluid therapy. Microsurgical instruments (Opitek, Denmark) and an operation microscope (Wild Heerbrugg; Heerbrugg, Switzerland) were used for the procedure. The animals' temperature was held at a constant of 37.0° by a temperature control unit (HB 101/2, Panlab Harward Apparatus, Stockholm, Sweden). The first 24 hours after operation the mouse cages were placed on heat plates (30°C).

Experiments I through VI (Table 1)

Prior to constructing the anastomoses, ischemia was induced by bipolar coagulation of blood vessels supplying the anastomosis. Varying numbers (2–4 of the smallest vessels and 1 large vessel) of supplying vessel were coagulated in order to create anastomotic leakage, since the extent of ischemia needed to create clinical impact was unknown (see Fig. 1). Thus studies I-III served as pilot studies to determine the proper amount of ischemia (see Fig. 2). This degree of ischemia was then evaluated in experiments IV through VI. However, ischemia was gradually increased throughout these three experiments due to absence of anastomotic leakage. It was impossible clearly to distinguish arteries from veins due to their small size and therefore both vessels were coagulated. For this procedure, microforceps with bipolar coagulation was used.

The primary outcome measure was anastomotic leakage manifested as fecal peritonitis or abscess formation. Fecal peritonitis was defined as feces in the abdomen leaking from the anastomosis, and an abscess was defined as a cavity in close relation to the anastomosis with communication to the lumen containing purulent matter. Often smaller collections of white tissue were present in relation to the anastomosis (e.g., adherent omentum), of which the nature was impossible accurately to determine. Thus, collections of white tissue smaller than 1/8 of the circumference of the anastomosis were therefore not regarded as abscesses.

Postoperatively, the animals were observed for 7 days. Weight and wellness scores were recorded daily. Wellness score (1–12, 12 being the best clinical condition) determines wellbeing of the animals and has been described previously.[10]

After 7 days—or before if the mice were considered too ill (based on clinical evaluation or a wellness score of 6 or lower)—the mice were re-anesthetized and killed. Hereafter, re-laparotomy was performed. The abdomen was evaluated for signs of abscesses or fecal peritonitis. The anastomoses from the mice completing the experiment were resected en-bloc and examined for breaking strength using a material testing machine (LF+; Lloyd Instruments, Fareham, UK) with an XLC10n loading cell as previously described.[11] The anastomotic tissue was placed in 2 clamps and pulled apart at a constant speed of 10 mm/minute. The breaking strength was derived from a load-strain curve produced by computer software (Nexygen; Lloyd Instruments, Fareham, UK). It was recorded whether the bowel broke at the anastomotic line or in adjacent tissue.

Except for induction of ischemia in the anastomoses, every detail about the experiments in this study were similar to the procedure used for the control group (8 suture anastomoses) of a previous

Table 1 Results from the different experiments

Experiment	Controls (n = 20)	I (n = 4)	II (n = 4)	III (n = 6)	IV (n = 19[a])	V (n = 10)	VI (n = 10)
Clinical leakage rate	0%	0%	50% (**$P = 0.022$**[b])	0%	10.5% ($P = 0.230$[b])	0%	0%
Anastomotic stenosis/obstruction (large bowel obstruction) rate	0%	100% (**$P < 0.001$**[b])	75% (**$P = 0.002$**[b])	16.6% ($P = 0.231$[b])	5% ($P = 0.486$[b])	0%	20% ($P = 0.103$[b])
Breaking strength, (Newton), median (range)	0.53 (0.40 − 0.73)	NR	NR	NR	0.41 (0.36 − 0.59) (**$P = 0.001$**[c])	0.45 (0.26 − 0.54) (**$P = 0.009$**[c])	0.38 (0.26 − 0.46) (**$P < 0.001$**[c])
Wellness score	–	NR	NR	NR	Comparable ($P = 0.438$[d])	Comparable ($P = 0.145$[d])	Comparable ($P = 0.059$[d])
Weight loss	–	NR	NR	NR	Comparable ($P = 0.397$[d])	Higher (**$P = 0.025$**[d])	Comparable ($P = 0.059$[d])
Number of vessels coagulated	–	5 (1 large vessel branching into 5 of the smallest vessels)	2	2	2	3	4
Site of the anastomosis (cm distal to the cecum)	–	1	4	1	1	1	1

NR: not recorded.

[a]Twenty mice were initially enrolled in this study; however, 1 died during the operation as a result of a failure in the anesthesia apparatus.

[b]Compared with controls, Fischer's exact test.

[c]Compared with controls, Mann-Whitney test.

[d]Compared with controls, Friedman's test on differences, significant results are in bold.

Fig. 1 Vessels coagulated in the experiments (arrows point to small vessels).

experiment.[12] Thus, these animals have been used as reference when comparing the outcome of the present study.

Statistics and ethics

Experiments I, II, and III served as pilot studies to determine the amount of ischemia needed to produce leakage. By carrying out these experiments, the appropriate degree of ischemia was determined, whereafter a sample size calculation was needed. The sample size calculations for experiments IV, V, and VI were based on the results from a previous study, where technically insufficient anastomoses were evaluated.[12] In this study, the leakage in the control group was 0% compared with 40% in the intervention group. Such a leakage rate is considered optimal in an experimental model, since such a high rate allows easy detection of beneficial interventions. We aimed to produce the similar leakage rate in the ischemic anastomoses and therefore the minimal relevant difference was set to 40%. Hence, 20 animals in each group were needed (alfa 5%, beta 20%).

For categorical data Fischer's exact test was used. For continuous data (breaking strength, not normally distributed) Mann-Whitney's test was used. Absolute values for breaking strength are expressed as median (range). In Fig. 3, weight loss is presented as median values. For differences in wellness score and weight loss over time Friedman's test on

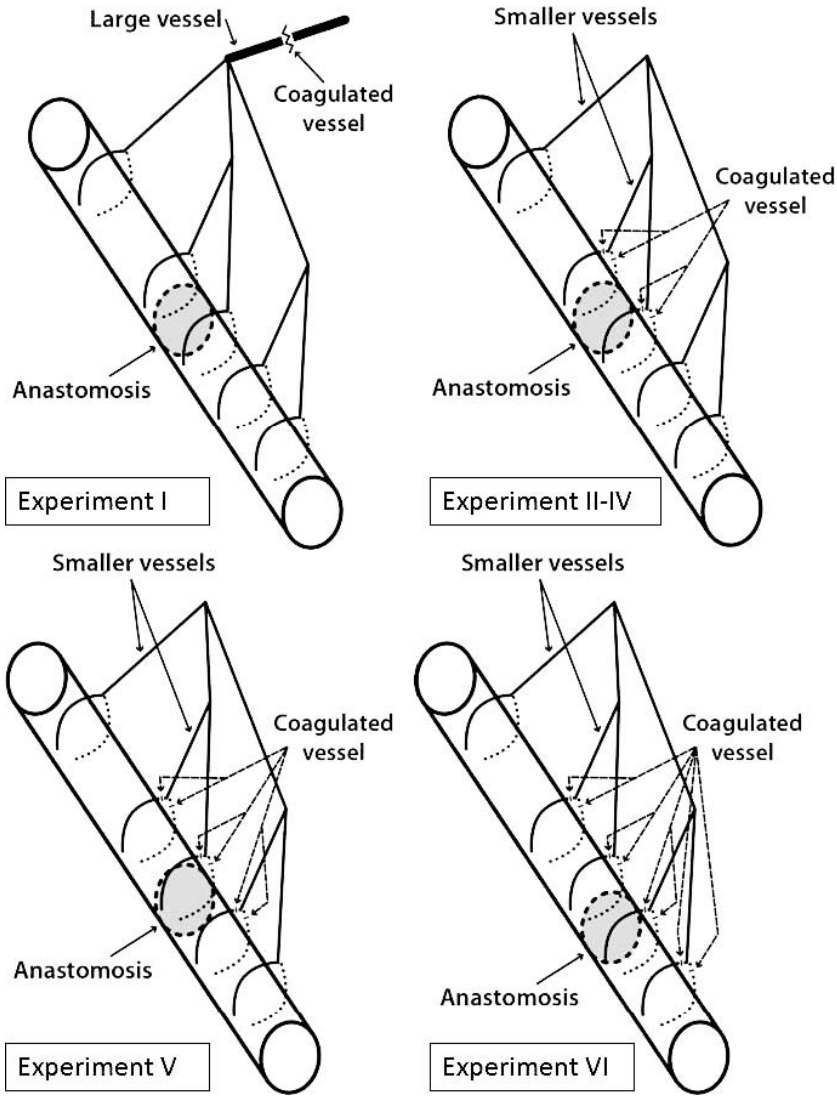

Fig. 2 The anatomy of vessels in relation to the anastomotic area and the place of coagulation in the different experiments.

differences in medians were used. Statistical software (SPSS version 19; SPSS, Inc., Chicago, Illinois) was used for the statistical analyses. P values <0.05 were regarded significant.

This study was approved by The Danish Council of Animal Experiments before initiation (license 2011/561-1977).

Results

Experiments I-VI

Breaking strength in all the anastomoses was significantly lower compared with controls (Table 1). However, a clear relationship between the

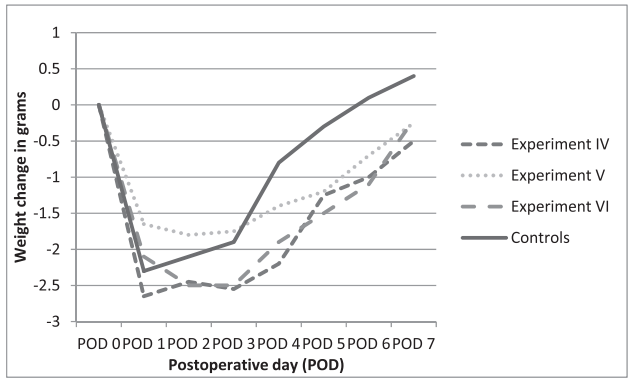

Fig. 3 The median weight loss in grams in the different experiment over PODs.

number of vessels coagulated and breaking strength in the anastomosis could not be detected.

As seen in Table 1, the clinical effect of ischemia was mainly large bowel obstruction. A clear tendency was that ischemia induced by coagulation of more than 3 small vessels resulted in an increased risk of large bowel obstruction. Moreover, a distal site of the anastomosis, as in experiment II, was associated with large bowel obstruction. Re-laparotomy in these animals revealed large bowel obstruction with massive small bowel dilatation caused by a stenotic/obstructed anastomosis.

Interestingly, more ischemia was not associated with increased risk of anastomotic leakage. Clinical leakage was seen in 2 experiments. In experiment II, a clinical leakage rate of 50% was observed. However, the majority of the animals (75%) had large bowel obstruction as well. In experiment IV, a clinical leakage rate of 10.5% was observed, which was not significantly different from the control group. Thus, the leakage rate was not high enough to conclude a relationship with ischemia. All animals with leakage had abscesses in relation to the anastomosis and none had fecal peritonitis.

As seen in Fig. 3, the course of weight loss for all the experiments was comparable with control animals until postoperative day (POD) 3, whereafter animals with ischemic anastomoses gained less weight. However, this difference was only significant for experiment V (Table 1).

The courses of wellness score were comparable between all experimental groups and controls (Table 1).

Discussion

The breaking strength of the anastomoses was significantly lower in all ischemic anastomoses, unrelated to clinical leakage rates and the degree of ischemia. The attempt to induce ischemia in the colonic anastomosis did not result in clinical leakage. Therefore, a functional model of ischemia as a pathophysiological mechanism for clinical leakage could not be created in the mouse model. Increasing ischemia by coagulating more vessels resulted in higher rates of large bowel obstruction, but without increasing clinical leakage rates. Except for in experiment V, the wellness score and weight loss were not significantly different between experiment and control animals.

The breaking strength was lower in all of the anastomoses with reduced blood supply compared with the control group, which may be a result of impaired healing of the anastomotic tissue. However, the healing impairment may have been insufficient to create clinical leakage. Given the possible multifactorial nature of anastomotic leakage,[13] this model may be used in future studier in combination with other known risk factors (e.g., steroid treatment),[14] where the individual contribution of several risk factors together may lead to anastomotic leakage, as seen in humans. Such models may add to our knowledge on relationship between and possible potentiating effects of such risk factors.

Few animals were included in the initial experiments (I, II, and III), since the consequences and clinical impact of the ischemic condition were unknown. Thus, it was considered unethical to include many animals. More mice were included in the following experiments, when the tolerated amount of ischemia was determined (reduced cases of ileus). Subsequently, more animals were included to create the necessary power to show a statistical difference between the groups. In experiment IV, 20 animals were included. However, in experiments V and VI, we did not find signs of leakage after evaluating 10 mice, but only cases of ileus. Therefore, we found it unethical to include the remaining 20 mice and the experiments were ended.

There might be several reasons for why it was not possible to create clinical leakage when compromising blood supply to the anastomosis. Anastomotic leakage most often has multifactorial reasons and occurs in sick patients with multiple comorbidities.[13] This model of ischemia creates pure ischemia in young, healthy animals. Hence, this model may be too simple compared with the situation in humans, where anastomotic leakage most often occurs. It is possible that these young, healthy animals may have a greater ability to produce angiogenesis, both in the bowel and from the surrounding adherent tissue. Adhesions may be able to create renewed perfusion by angiogenesis and may cover minor leakages at the anastomotic line. These factors stand in contrast to patients with comorbidities, universal atherosclerosis, and microvascular disease, who may have a decreased ability to restore perfusion to ischemic tissue. Moreover, there might be different pathophysiological mechanisms evaluated in this model where larger vessels are coagulated, compared with high risk patients who suffer from microvascular disease.[1–3] Another reason could be a technical issue since the vessels in this model were divided with bipolar coagulation. However, the mesentery of bowel was not transected with scissors after the coagulation was done. Therefore, the possibility exists that some of the

smaller vessels had regained blood flow. This may also explain why results in experiment I and VI were so different, when the apparent number of vessels without blood supply was almost similar. The large vessel, which was coagulated in experiment I, may have had nonvisible branches aside from the 5 branches which were observed, making ischemic insult stronger. However, it did not seem possible to induce more ischemia than done in experiment VI in any other way, since no more small vessels were available for coagulation. Another reason for the lack of anastomotic leakage may be that the stenotic anastomosis created by ischemia limits the amount of feces that is able to pass through the anastomosis and thereby the risk of leakage. Moreover, it is known that many leakages in humans occur around the 7th postoperative day.[15] Since this experiment ended 7 days after surgery, the possibility exists that more leakage may have occurred if the experiments had lasted longer (e.g., 21 days). The use of a control group from a prior experiment was not considered a problem for the experimental design, since the prior experiment was executed just before the present study. Therefore, the operative skills of the investigator were assumed to be unchanged. In this line of reasoning, the failure to create clinical leakage in these anastomoses was not considered a result of better technical abilities. Supporting this was the leakage and large bowel obstruction rate of 0% in the control group, which rendered the technique optimal at that time. Although a proper randomization would have given a better experimental design, this is less relevant when constructing a model of ischemia and not evaluating an effect of a treatment compared with placebo. As a result, we found it unethical to include a new control group.

Other studies evaluating isolated ischemia and anastomotic leakage have also failed in creating clinical leakage. Two studies have been carried out in rats where anastomotic ischemia was induced. In one study, ischemic anastomosis was created in the rat by ligating arteries in 3 cm of anastomotic mesentery, but sparing the marginal artery.[4] Blood flow was substantially decreased to less than 10% of baseline, but there was no significant correlation between reduced blood flow and bursting pressure in the animals. Colonic obstruction and distension of the colon was reported in 46% of the animals and clinical anastomotic leakage was found only in 7.7% of the animals. However, there was no control group for comparison. In another study using rats, 2 cm of mesentery on either side of the anastomosis were divided on the left colon.[5] Corresponding to our results, this study found reduced healing, as measured with bursting pressure, in ischemic anastomosis compared with the control group and no significant difference in clinical leakage. However, the study did not report colonic obstruction in any of the animals. In another study carried out in pigs, ligation of supplying vessels in 5 cm of the mesentery on either side of the colonic anastomosis was done.[7] However, a leak of 18 mm was applied to the anastomosis at the same time. Despite clinical leakage in the control group, none of the animals with maximum ischemia had clinical leakage. Instead, large bowel obstruction was reported in 75% of the cases. The abovementioned studies correlated well with our findings, suggesting that pure ischemia in the experimental animal model may induce large bowel obstruction due to colonic stenosis or obstruction and not clinical anastomotic leakage as intended. On the other hand, a study carried out in dogs found clinical leakage in 70% of the cases without any obstruction or sign of large bowel obstruction.[6] However, this model used a combination of ischemia and technically insufficient technique. Hence, it cannot be ruled out that the clinical leakage was a result of the technical insufficiency and that the degree of ischemia may have been too small to create obstruction.

In conclusion, we found that ischemia as induced in this study did not create clinical leakage in mice. In contrast, large bowel obstruction was observed in the animals with the highest degree of ischemia. Breaking strength was significantly reduced in the ischemic anastomoses, which may correlate with the reduced healing of the tissue. At this point, is seems that pure ischemia in the experimental setting is too simple a setup to create clinical leakage. This model may be used in combination with other known risk factors for anastomotic leakage, to mimic the multifactorial origin of anastomotic leakage in humans.

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