ONLINE Material and Methods

The colonoscopic leakage model: a new model to study the intestinal wound healing at molecular level

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Animals

All mouse experiments were approved by the Ethical Committee for Animal Care of the Health Sector of the Université catholique de Louvain, under the supervision of Prof. JP Dehoux under number 2018/UCL/MD/013 and were performed in accordance with the guidelines of the Local Ethics Committee and in accordance with the Belgian Law of May 29, 2013, regarding the protection of laboratory animals (agreement number LA1230314).

For all experiments, male C57BL/6J mice were purchased from Janvier (Le Genest-Saint-Isle, France) and were housed in pairs under strict specific and opportunistic pathogen free (SOPF) conditions and in controlled environment (room temperature of 22 ± 2 °C, 12 hour daylight cycle, lights off at 6 pm) with free access to food and water.

In all our procedures, we abstained from using antibiotics either pre- or post-intervention.

Adapted Anastomotic Leakage Model

More and more studies suggest that the mouse is the most suitable animal to realize an anastomotic leakage model [1]. We decided to implement the anastomotic leakage model proposed by Pommergaard [2, 3] in our facility.

Briefly, this murine model of colonic anastomosis after short resection, aims to obtain a postoperative probability of 50% of anastomotic leakage and peritonitis. This is achieved by varying the number of surgical sutures used to perform the anastomosis, with either 4 separate points for the "peritonitis-group" or an 8 separate point colonic anastomosis for the "control-group" in which no intra-abdominal infections are expected. The only difference between the two groups are the gaps between two adjacent stiches or, in other words, the size of the five transmural wound which are present between each two adjoining stiches. The resections/ anastomosis is done in the left colon, below the left colic angle. The colonic vascularisation is preserved, and the anastomosis is done tensionless.

In our laboratory, we made use of a surgical trinocular with 20-40 X magnification with the possibility of filming through the extra optic (Nikon SMZ800N). Microsurgical instruments (needle holder, scissors and forceps) were purchased from FST (Fine Science Tools, Heidelberg, Germany) and special attention was paid to choose an adequate atraumatic suture tying forceps in order to avoid involuntary perforations when handling the intestines.

Our procedure went as follow:

Anaesthesia was performed using isoflurane gas (Forene, Abbott, Queenborough, Kent, England), in accordance with welfare criteria [4]. We used a dose of 3% for induction and of 2,5 % during the intervention. Mice were placed on a heated plate to ensure the body temperature did not drop too low.

After gently pinching the hind paw and confirming the loss of reflex, the abdomen was then disinfected and a midline skin incision of 2 cm was made with scissors (better than using scalpel) in the middle third of the abdomen, after which, the muscular abdominal wall was lifted away from the intestine in order not to injure any intra-abdominal organs and cut at the midline, between the two rectus abdominis muscle, in a complete avascular plane.

An intraperitoneal injection of buprenorphine 0.3mg /ml diluted 100 times (Buprecare; Animalcare Limited, UK) with the µl-equivalent of 6 times the weight of the mouse was administered.

The caecum and rectum were used as reference points to locate the site intervention, about one centimetre caudal of the left colic flexure. The resection segment should not be larger than 2-3 mm, in order not to put too much tension on the future anastomosis. Particular attention should be paid on the mesocolic vascularisation and the resection segment should be chosen in a zone between two vascular branches, in order note to induce any bleeding or impair the vascularisation of the two colon edges.

It is also important to avoid manipulating the intraabdominal structure with graspers when searching for the site of intervention, as this could cause unwanted damage. It is preferable to use sterile nasal cotton plugs for this purpose. Also, the intraabdominal organs should not be taken out of the abdominal cavity in order to create working space but kept in the abdominal cavity which should be moistened at regular intervals using saline solution.

The segmental resection of the colon is performed using scissors, taking care not to damage any blood vessels. The end-to-end colo-colic anastomosis is done in *Nardi* technique using 8-0 Vicryl® interrupted inverted sutures. The stiches need to be placed at equal distances, especially if doing 5-point anastomoses. Applying first stich at the mesenterial level simplifies the rest of the procedure. If doing an 8-point anastomosis, the second stich should be placed at the anti-mesenterial level, followed by three stiches at equal distances on both anterior and posterior faces.

The tightness of the anastomosis can be verified directly postoperatively, before closing the laparotomy wound, either by covering the anastomosis with saline solution and insufflating air rectally, or by using a solution of Methylene blue 1% (Neopharma, Aschau, Germany) that was introduced via a flexible rectal probe. The latter was our method of choice as it allowed better visual control and was easier and faster (see online Videos showing the presence of the absence of leakage).
Beside the tightness, we checked also for lack of stenosis in the anastomotic region. This can be done either by using a colonoscope with a 3mm endoscopic sheath or by passage of a 20 Ga x 30 mm disposable plastic feeding tubes through the anastomosis.

After verifying the tightness and lack of stenosis, the abdominal wall is closed using a 4-0 Vicryl® running sutures, followed by closure of the skin using a running 5-0 Byosine™ suture. At the end of the intervention, 150 µl of Ketofen 10%, 100mg/ml diluted 100 times (Ketofen; Ceva Sante Animale, France) was administered Intraperitoneally.
The average intervention time was 45 minutes per mouse. The first two hours after surgery, mice were placed in a climate chamber set at 25°C to improve recovery. Mice that underwent the procedure were observed twice a day and their well-being was assessed using the wellness score proposed by Komen [5]. They were weighed once a day.

Painkillers were administrated for four days after intervention. Postintervention analgesia was multimodal, using a combination of paracetamol and NSAIDs (Ketoprophene; Merial, Belgium) administered subcutaneously, for 48 hours postoperatively, followed by Paracetamol alone, administered orally (in the drinking water), for another 48 hours. Under these conditions, postoperative pain was estimated to be moderate.

After 7 days, mice were anesthetized with isoflurane. Evaluation of the severity of the peritonitis using our Peritonitis Score was done by two different investigators which were blinded to the mouse groups. The concordance between the two evaluators was 100%.

Evaluation of the Pommergaard-Adapted Anastomotic Leakage Model

In our hands, the 50 % probability of anastomotic leakage and peritonitis was obtained when performing a 5 separate point colonic anastomosis, as four suture points almost always resulted in leakage.

As also remarked by Pommergaard, one of the most important shortcomings of this model is the occurrence of large bowel obstruction. This is caused on the one hand by the surgical traumatism at the level of the anastomosis, and on the other hand by the peri- and postoperative analgesic regimen used during the recovery period, as the administered mixture is known to impact on bowel movement and transit time [6]. Neither of these causes can be eliminated, as surgical trauma and reduced flexibility is inherent to the procedure and the perioperative administration of opiates is mandatory for the well-being of the animals.

Contrary to rodent bariatric surgery models, in which provision of liquid nutrition during the perioperative period is standard practice [7], supplying liquid diet (Liquid Diet, AIN-76, Bio-Serv, Flemington, NJ) did not minimizes the risk of postoperative intestinal stasis and obstruction in our experiments. We did not observe an effect of the liquid diet on body weight loss, but coagulation of the liquid diet, even within specially adapted delivery systems, made it very impractical, reason why we chose to continue to use our standard diet throughout the rest of the experiment.

After performing different pilot studies in order to fine-tune the experimental set-up and achieve similar results as those described by Pommergaard, we started an experiment in which 11-weeks old male C57BL/6J mice were randomly assigned to one of three experimental groups: two groups underwent anastomosis with either eight (n=8) or five (n=7) separate sutures, the third group (n=6) had a sham laparotomy without intestinal resection/anastomosis.

In the sham laparotomy group, we did not observe any peritonitis or weight loss. The mice displayed no signs of discomfort or sufferance and there was nothing to indicate their bowl movement was affected. In the anastomotic groups we had a general body weight drop of over 15% with little to no recovery after 5 days.
We obtained 53% peritonitis in the anastomotic leakage group with 5-point anastomosis and 12,5% peritonitis in the control group with 8-point anastomosis, indicating this model is feasible and reproductible.
However, we also found sub-occlusion in 53% of the animals (excluding the sham group) and even observed complete bowel obstruction with massive dilatation of the small intestine in some animals (20%). The occlusions were most prevalent in in the Anastomotic Leakage group (63% in animals with 5 sutures) and the rest in the control group (8 sutures) and were not always corelated with the appearance of a local or general peritonitis.

These outcomes were incompatible with our main experimental purpose which is to study the intestinal wound healing at molecular level while being able to evaluate the impact of different local manipulations.
Moreover, this model is very time-consuming, making it difficult to obtain sufficient animals per experimental group, especially since the drop-out rates are high due to the high occurrence of intestinal obstruction.

Taking all these elements into account, we set out to develop a better, more reliable and more efficient model of intestinal wound healing in the mouse.

We noticed that the gap between two adjacent stiches in the case of a 5-point anastomosis is more or less equal to the opening of our 3Fr. colonoscopical grasper. With this in mind, we imagined a model of endoscopic transmural perforation, in which a single puncture was created at a precise location during a simple colonoscopical examination. We termed this the "*colonoscopic leakage model*". This model was designed to resemble clinical reality of small intestinal perforation as is the case with perforated diverticulitis and iatrogenic colonoscopic perforations.

Colonoscopic leakage model

This procedure necessitates a white light colonoscopic system designed for use in rodents, with a monitor for easy visualization of the colon throughout the procedure (for references of the system, see below). We used a system combining a high-resolution camera with a rigid examination sheath that enables local manipulation through an endoscopic sheath working channel.

The feasibility of practicing rigid colonoscopies in mice has been described in 2006 by Becker and al. [8] and it has since then been used mostly to asses colonic dysplasia development [9] or the inflammatory conditions in colitis [10], though never in the context of transmural wound healing.

One of the advantages of using this technique, is that the mice do not need a preoperative bowel preparation, nor do they need to be fasted before the intervention. In fact, contrary to observations on rats [11], our pilot experiments showed that an overnight or even a 24-hours fasting is not sufficient to completely empty the intestines and provide adequate bowel preparation. However, in order to perform colonoscopical examinations in optimal conditions, we suggest flushing the colonic lumen to remove any faeces that may hamper visibility or prevent access to the site of interest. To do so, we suggest using flexible soft-tip feeding needles to administer a trans-anal enema with 1 ml of saline solution 10-15 minutes before the intervention. This has proven efficient in ensuring a colonoscopical-clear view

without residual gut content up to the left colonic angle. Of note, performing this enema too early (one or two hours before the procedure) was not effective.

The correct adjustment of the CO_2 flow is of crucial importance. A CO_2 flow rate of 0,4 L/min and a working pressure set to 3 mmHg was optimal to obtain sufficient insufflation of the colon without creating an overpressure, that may in certain cases lead to suffocation of the animals. Indeed, we observed that augmenting the pressure to 6 mmHg resulted in an important incidence of peri-interventional fatalities caused by a massive dilatation of the stomach. Similar problems were encountered when trying to improve visibility by flushing the intestines during the intervention with volumes of saline solution larger than 1 ml.

The procedure includes the following steps:

Anaesthesia was performed as described above (3% induction and of 2,5 % maintenance dose). There is no need to place mice on a heated on a hot plate as this is a fast and non-invasive procedure. The examination sheath should be introduced gently and about 1 cm from the anal verge. After fitting optical fibre and the camera, the examination sheath can be guided through the colon until just before the left colic flexure, i.e. 3,5 cm ab anno and lateral to the mesocolon. We do not recommend the use of any lubricant for the introduction of the colonoscope, as this may blur the image if smeared on the optic fibres. Marking the sheath with a reference point, makes it easier to standardize the location.

3Fr-biopsy forceps can then be introduced through the endoscopic sheath working channel. The transmural wounding is performed by opening the forceps at the desired angle, pressing it against the colonic wall, grasping and pulling while twisting. The biopsy obtained this way can be collected and snap-frozen for further analysis, if needed.

Successful perforation is immediately objectivated by the manifestation of a pneumoperitoneum with instantaneous inflation of the abdominal cavity. If not maintained for too long time, this has no impact on the wellbeing of the mouse. The deflation of the peritoneal cavity is achieved by intraperitoneal introduction of a needle (19G) for a few seconds to allow excess gases to escape.

Mice can then be returned to their habitual environment, there is no need to keep them in a climate chamber.

Because this is a fast procedure, maximum efficiency is achieved when performed by three persons: one is in charge of managing the animals (weighing and flushing the animals prior to the procedure, timing initial anaesthesia and returning animals to their cages), one is doing the colonoscopy and maintains the correct positioning, while a third person manipulating the grasper, performs the actual perforation and collects the biopsy.

The entire procedure takes less than 10 minutes (i.e., 6 times faster than the anastomosis model). Additional advantages include very low peri-interventional mortality and fast recovery of animals, with mice displaying normal behaviour as soon as the anaesthetics wear

off, eliminating the necessity of using high doses of opiates. Weight loss is also very limited, and mice return to their pre-operative body weights in less than 5 days. Importantly, we did not observe any signs of intestinal occlusion, indicating the procedure does not impair normal bowel movement.

If all the precautions described above are considered, this method generates a percentage of peritonitis of 40-50 % during the first 7 days post-intervention. This is similar to the results obtained in the anastomotic leakage model, but without all the aforementioned shortcomings.

The mortality rate before the selected endpoint (i.e. 7 days) is between 10 -19%, which is much lower than that of the anastomotic leakage model. Moreover, all of these were due to either a localized or generalized peritonitis.
Because we observed distinct gradations of inflammatory response within the animals, we developed a proposal for the use of a peritonitis score, which defines five end-point categories groups:
0) mice in which no peritonitis was observed,
1) mice with a peritonitis that was localized only to the site of perforation and that did not result in death,
2) Mice with a generalized peritonitis affecting the whole abdominal cavity, but that did not result in death,
3) mice that died before the end of the 7-day period that had only a localized peritonitis and
4), mice that did and displayed clear signs of a generalized peritonitis.
All categories were given a specific score to allow a more sensitive appreciation of the outcome and a fast evaluation of comparative experimental designs. The reproducibility of this scoring was confirmed independently by two different, blinded examiners without any difference in the evaluation.

Score	**Groups**
4	Fatal general peritonitis
3	Fatal, but localized peritonitis
2	Non-fatal general peritonitis
1	Non-fatal localized peritonitis
0	No peritonitis

At necropsy, blood and tissues can be sampled as usual. In addition, the site of perforation can be identified and prepared for protein/RNA extraction or for histological analysis. We recommend using a surgical trinocular.

Equipment and Materials:

1. **Colonoscopic tower**: Telepack X Video Unit/ portable all-in-one system from Karl Storz, Tuttlingen, Germany (with possibility of digital recording included)
2. **Insufflator**: Karl Storz Electronic human CO2 Endoflator 26430520 set up with a CO_2 flow rate set to 0,4 L/min and a working pressure set to 3 mmHg
3. **Optics**: HOPKINS rigid Telescope, 1.9 mm, Length 10 cm
4. **Camera:** Karl Storz TELECAM One-Chip Camera Head, PAL, F = 25-50mm
5. **Light Cable**: Fiber Optic Light Cable, Ø2.5mm Karl-Storz
6. **Endoscopic Sheath:** either a protect and examination sheath 7 Fr. Length 8,5 cm without presence of a working channel or an examination sheath 9 Fr. Length 7 cm with presence of a working port.
7. **Grasper:** Biopsie Forceps3 Fr.

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