Video Article

Functional Assessment of Intestinal Motility and Gut Wall Inflammation in Rodents: Analyses in a Standardized Model of Intestinal Manipulation

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Abstract

Inflammation of the gastrointestinal tract is a common reason for a variety of human diseases. Animal research models are critical in investigating the complex cellular and molecular of intestinal pathology. Although the tunica mucosa is often the organ of interest in many inflammatory diseases, recent works demonstrated that the muscularis externa (ME) is also a highly immunocompetent organ that harbours a dense network of resident immunocytes.[1,2] These works were performed within the standardized model of intestinal manipulation (IM) that leads to inflammation of the bowel wall, mainly limited to the ME. Clinically this inflammation leads to prolonged intestinal dysmotility, known as postoperative ileus (POI) which is a frequent and unavoidable complication after abdominal surgery.[3] The inflammation is characterized by liberation of proinflammatory mediators such as IL-6[4] or IL-1β or inhibitory neurotransmitters like nitric oxide (NO).[5] Subsequently, tremendous numbers of immunocytes extravasate into the ME, dominated by polymorphonuclear neutrophils (PMN) and monocytes and finally maintain POI.[2] Lasting for days, this intestinal paralysis leads to an increased risk of aspiration, bacterial translocation and infectious complications up to sepsis and multi organ failure and causes a high economic burden.[6]

In this manuscript we demonstrate the standardized model of IM and *in vivo* assessment of gastrointestinal transit (GIT) and colonic transit. Furthermore we demonstrate a method for separation of the ME from the tunica mucosa followed by immunological analysis, which is crucial to distinguish between the inflammatory responses in these both highly immunoactive bowel wall compartments. All analyses are easily transferable to any other research models, affecting gastrointestinal function.

Video Link

The video component of this article can be found at http://www.jove.com/video/4086/

Protocol

1. Animals

Male C57Bl6/J mice with a mean weight of 25 g were obtained from Harlan Winkelmann (Borchen, Germany). All experiments were performed in accordance with the federal law regarding the protection of animals. The principles of laboratory animal care were followed. Animals were maintained on a 12-hr light/dark cycle and provided with commercially available rodent chow and tap water *ad libitum*. Animals should be housed at least 7 days after arrival in your animal facility before experiments. *Note that all experiments can be easily adapted to rats or other species and with slight modifications into large animal models.*

2. Operative Procedure

1. Anesthesia is induced and maintained with isoflurane (Abbott, Wiesbaden, Germany) in oxygen. After onset of anesthesia place animals in supine position and fix extremities with adhesive tape (Leukosilk, Beiersdorf, Hamburg, Germany).
2. After shaving and surgical disinfection of the abdominal wall a median laparotomy is performed on a length of 2 centimeters. Enter the abdominal cavity via an incision along the Linea alba. Place two sterile retractors to keep the abdomen open. Carefully eventerate the small bowel to the left and place it on a wet cotton gauze.
3. Evacuate the entire small bowel luminal contents with two moist and sterile cotton applicators (MaiMed, Neunkirchen, Germany) by simultaneously rolling the applicator from the pylorus to the cecum. CAUTION: Avoid hemorrhages of the small bowel wall or lesions of the gut vessels. Put the gut back into the peritoneal cavern without twisting to prevent ischemia or mechanical obstruction and close the abdominal incision using a two layer continuous 5/0 polyamid non-absorbable sutures (Ethicon, Norderstedt, Germany).

3. Postoperative Care

1. After the procedure place the animal under a heating lamp, observing it until it recovered from anesthesia. Put the mouse back in its cage and allow access to water and food. Due to the property of opioids to affect peristalsis, we perform perioperative analgesia with NSAIDs (for example metamizol) in drinking water.

4. Functional Studies 24 hr after IM

a. *Gastrointestinal Transit (GIT)*
   1. Anaesthetize mice slightly with isoflurane in oxygen 22.5 hr after IM. Carefully hyperextend the head of the mouse and pull out the tongue with a blunt forceps. Fill a 1 ml syringe with at least 100 µl fluorescein-labeled dextran (FITC-dextran, 70,000 MW, Sigma Aldrich, Taufkirchen, Germany), connect it to an arterial catheter (Vygon, Ecouen, France) and carefully insert the catheter as a gastric tube into the stomach.
   2. Briefly inject 100 µl FITC-dextran and pull out the catheter. Let the animal awake and wait for 90 min. During this time mice have access to food or water.
   3. Prepare fifteen 2 ml spinning tubes and label them consecutively.
   4. Euthanize mice 90 min after gavage and remove the entire gastrointestinal tract from stomach to rectum. Place the intestine onto a polystyrene pad and avoid stretching. CAVE: It is crucial to remove the gut gently to avoid shifting of liquid FITC-dextrane into the nearby segment. For the beginning it might be helpful to use clips at the beginning and at the end of each segment.
   5. Measure full length of small bowel and full length of colon. Divide the small bowel into 10 equal sized segments by pinning it down to the polystyrene pad with cannulas. Divide the colon into 3 segments. You have prepared 15 segments (stomach #1), ten equally sized small bowel segments (#2-11), cecum (#12) and three 3 equally sized colonic segments (#13-15).
   6. Transect the intestine at the marked positions, grab it with a forceps and flush them once with 1.0 ml KHB into the previously prepared KHB containing 2.0 ml tubes and vortex vigorously for 20 sec. The stomach and cecum are placed directly and cut in the prepared tubes. Fill these tubes with 1.0 ml KHB, thereby rinsing traces of luminal contents from the scissor into the tubes.
   7. Centrifuge the probes at 12,000 rcf for 5 min at room temperature in a table top centrifuge. During centrifugation prepare another 15 consecutively numbered 1.5 ml tubes.
   8. Transfer the clear supernatants into the new 1.5 ml tube and store in the dark at 4 °C until analysis (next step). The probes can be stored for several days at 4 °C without significant loss of FITC signal. For longer storage add 0.09% Natrium-azide or store at < -18 °C.
   9. Pipette 100 µl duplets of the supernatants on a black 96-well plate (Greiner Bio One, Frickenhausen, Germany). Pipette two 100 µl samples of KHB as blank.
   10. Read the plate in a fluorescence reader (Safire, Tecan, Crailsheim, Germany) and quantify the fluorescence at 494 nm (absorption) / 521 nm (emission) wavelength. Subtract blank values from samples.
   11. Calculate the geometric center (GC) of FITC-dextran distribution, which is actually the center of gravity for the distribution of marker, by the following formula: GC = ∑ (% of total fluorescent signal per segment * segment number) / 100

   Note: By multiplying percentage of fluorescence of each segment with its segment number a weighted mean of the distribution of marker within the intestine is assessed. This point is influenced by both the distribution and distance traveled by marker, but assumes no specific underlying distribution.[7] The calculated GC value is often presented in combination with a graph demonstrating the distribution of FITC dextran over the gastrointestinal tract (**Figure 1A**).

b. *Colonic Transit*
   1. Mice should be weakly anaesthetized with isoflurane 2 % in oxygen. CAUTION: Ensure that the animal will awake within 40 sec.
   2. Carefully examine colonic patency by inserting a fistula probe (metal rod, 2 mm diameter) through the anus into the colon.
   3. Using the fistula probe push forward a 2 mm glass ball 3 cm into the colon and pull out the probe immediately. Place the mouse in a transparent cage and start measuring the time after the fistula probe was pulled out of the colon. Keep an eye to the mouse and stop the time directly after the glass ball became excreted.

5. Histochemical Analysis of Isolated ME Specimens

1. Midjejunal segments are cut from the bowel and immersed in cold KHB in a Sylgard-filled dish.
2. Mesentery is pinned to the dish with 0.2 mm insect needles (Fine Science Tools, Heidelberg, Germany).
3. Cut open the segment along its mesenteric border and stretch to ~120 % of its length and width. Finalize the fixation with insect pins on opposite site of the mesentery. Rinse the sylgard dish carefully with fresh ice cold KHB, thereby flushing all luminal contents.

   ME can now mechanically separated from the tunica mucosa starting from the mesentery. During preparation replace fresh chilled KHB several times.
4. Separated tunica mucosa can be shock frozen in liquid nitrogen for further analysis or discarded.
5. Fix the isolated ME whole mount with 100 % ethanol for 10 min at room temperature.

   Wash the whole mount three times using chilled KHB. Note: other fixatives (*i.e.* 4% formaldehyde, acetic acid, aceton) can also be used after testing its compatibility with the subsequent analysis.
6. The ME whole mounts are ready for further histochemical or immunhistochemical staining:

a. *Histochemistry: myeloperoxidase (MPO) staining*

   Infiltrated PMNs can be examined by the following MPO staining protocol.

1. Solve 10 mg Hanker Yates reagent (Polyscience Europe, Eppelheim, Germany) in 10 ml KHB and add 100 µl of 3% H_2O_2. Prepare the solution freshly immediately before use and do not reuse.
2. Incubate whole mounts for 10 min at room temperature.
3. Rinse specimens extensively with cold KHB and incubate for 10 min.
4. Mount specimens in aqueous mounting medium (Aquatek, Merck, Darmstadt, Germany) After drying for 2 hr analyze slides at under a light microscope.
5. Count numbers of cells in 5 randomly chosen fields and calculate as MPO positive cells / mm^2.

b. *Immunohistochemical Staining:*

Accordingly to the histochemical MPO staining, immunohistochemical stainings of the whole mounts can be performed. Principally, your established immunohistochemical staining protocols of cell cultures or tissue slices can be easily adapted to a whole mount staining. However, optimizing the procedure for specific antibodies is strongly recommend.

1. Cut specimens into 5 x 5 mm pieces and perform staining in 2.0 ml round bottom centrifuge tubes in ~150 µl - 200 µl antibody solution.
2. Fixation and blocking procedure (*i.e.* 3% BSA in PBS for 1 hr at room temperature) can be performed in the "sylgard dish".
3. Washing steps in PBS or other buffers can be performed in 12-well plates.
4. Transfer the .specimens carefully with a sharp forceps between staining tubes and washing plates.
5. After the final washing procedure mount specimens in anti-fading mounting medium with cover slips and analyze with a fluorescent microscope.

6. Organ Culture of ME

1. The whole small intestine is resected 24 hr after IM and placed in cold KHB containing 200 U/ml penicillin G and 100 µg/ml streptomycin (KHB + P/S).
2. Cut the gut into 3 cm lengths and pin each segment down to a Sylgard containing glass dish.
3. Remove the mesentery with fine scissors and slip the intestine on a knitting needle.
4. Gently incise the ME with a sharp forceps and strip it off from the submucosa using a moist cotton applicator. The tunica mucosa remains on the knitting needle and can be snap frozen for further investigation. The ME is collected in cold KHB + P/S.
5. Cut the isolated ME strips into small pieces of 2 - 4 mm length and incubate it in ice cold KHB + P/S for half an hour and rinse the specimen several times.
6. Transfer approximately 50-60 mg ME (at least half ME of one small bowel) in a sterile 24-well plate containing 500 µl Dulbecco s modified Eagle medium (DMEM).
7. Incubate at 37 °C and 5 % CO_2 for additional 24 hr.
8. Inspect the cell cultures for bacterial or fungal contamination under a microscope.
9. Collect supernatant, spin down for 5 min at 1,000 rcf and snap freeze in liquid nitrogen. Meanwhile blot dry the muscle tissue on a clean tissue for 30 sec and weight.
10. Supernatants can be used for subsequent analysis of released cytokines (IL-6) by ELISA or other metabolites (*i.e.* nitrite) in the supernatant by following manufactures instructions.
11. Normalize measured concentrations per weight of ME tissue.

7. Representative Results

1. Functional Studies

The most important parameter to evaluate the severity of POI is the examination of GIT *in vivo*. **Figure 1A** demonstrates a typical distribution of FITC dextran along the gastrointestinal tract in untreated controls and intestinally manipulated mice 24 hr after surgery. "Sham operated" animals showed a normal GIT with a calculated geometric center (GC) in the cecum while IM led to a significant delay of GIT in the proximal jejunum (**Figure 1B**)

Colonic motility was separately focused on by measurement of the ball excretion time of a 2 mm glass ball. Sham operated mice show an excretion time of 48 - 200 sec while IM led to a prolonged excretion time between 470 - 775 sec (**Figure 1C**).

2. Morphological Studies

To describe the inflammatory extend within the ME several phenotypes of leukocytes could be analyzed using histochemistry or immunohistochemistry. In **figure 2A+B** a low presence of PMNs in "sham operated" animals was observed (39±7 cells/mm^2). IM of the small bowel led to a strong PMN infiltration (660±86 cells/mm^2) compared with sham operated mice

3. ME Organ Culture

Many inflammatory mediators liberated from cells are difficult to detect in tissue lysates. Organ cultures allow detection of liberated mediators in the culture supernatant. A representative mediator in POI is NO, which is as major inhibitory neurotransmitter in the gastrointestinal tract.[5]

NO could not be detected in ME organ culture supernatants of unoperated control animals (5±6 µM/ 24 hr /mg tissue). In sham operated (laparotomy) mice basal NO levels of 53±36 µM/ 24 hr /mg tissue were observed. ME cultures of intestinally manipulated (harvested 24 hr after surgery) mice produce significantly more NO (2254±853 µM/ 24 hr /mg tissue) during 24 hr ME organ culture (**Figure 3**).

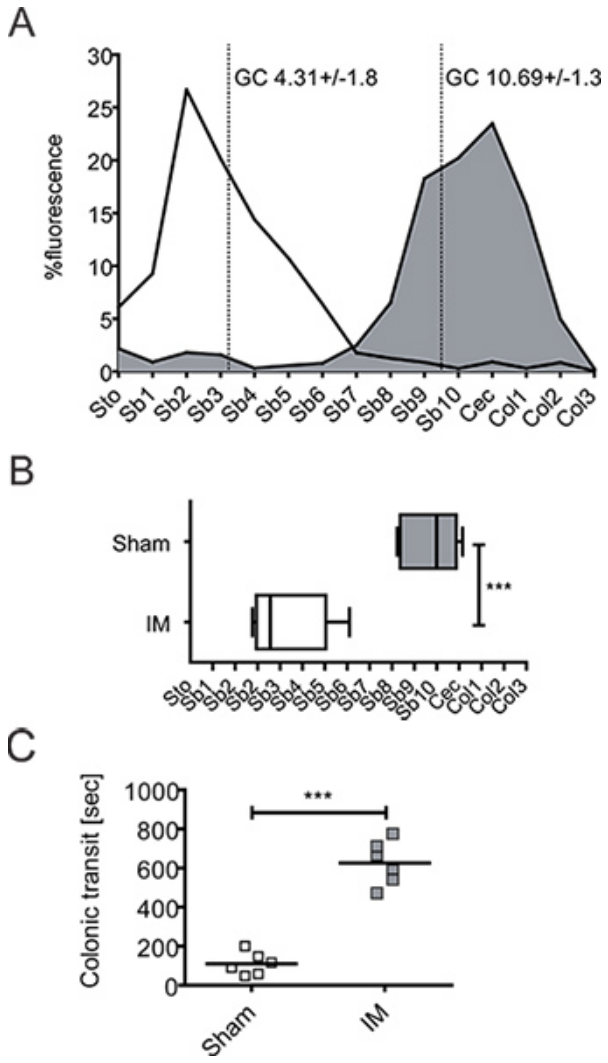

Figure 1. Effect of IM on GIT (*A+B*) and colonic transit (*C*) GIT was measured as the percent of non-absorbable fluorescein-labeled dextran in 15 gastrointestinal segments-stomach (Sto), small intestine (SI 1-9), cecum (Cec), and colon (Co)-90 min after oral ingestion. Colonic transit was measured as the time from pulling out the fistula probe until excretion of a 2 mm glass bead. *(A)*Gastrointestinal distribution of fluorescein isothiocyanate- dextran after sham operation or IM. In sham operated animals most of the marker is located in the cecum or the proximal colon compared with proximal jejunal location in manipulated animals. Dotted lines indicate calculated GC. (B) Calculation of the GC demonstrated a prolonged gastrointestinal transit time after IM. (C) Colonic transit time showed a significant delay in IM mice compared with sham operated animals. GC for the 15 intestinal segments are displayed as mean (n=5). Colonic transit times were displayed mean with all individual values (n=6). *** = p<0.001, Student's t-test.

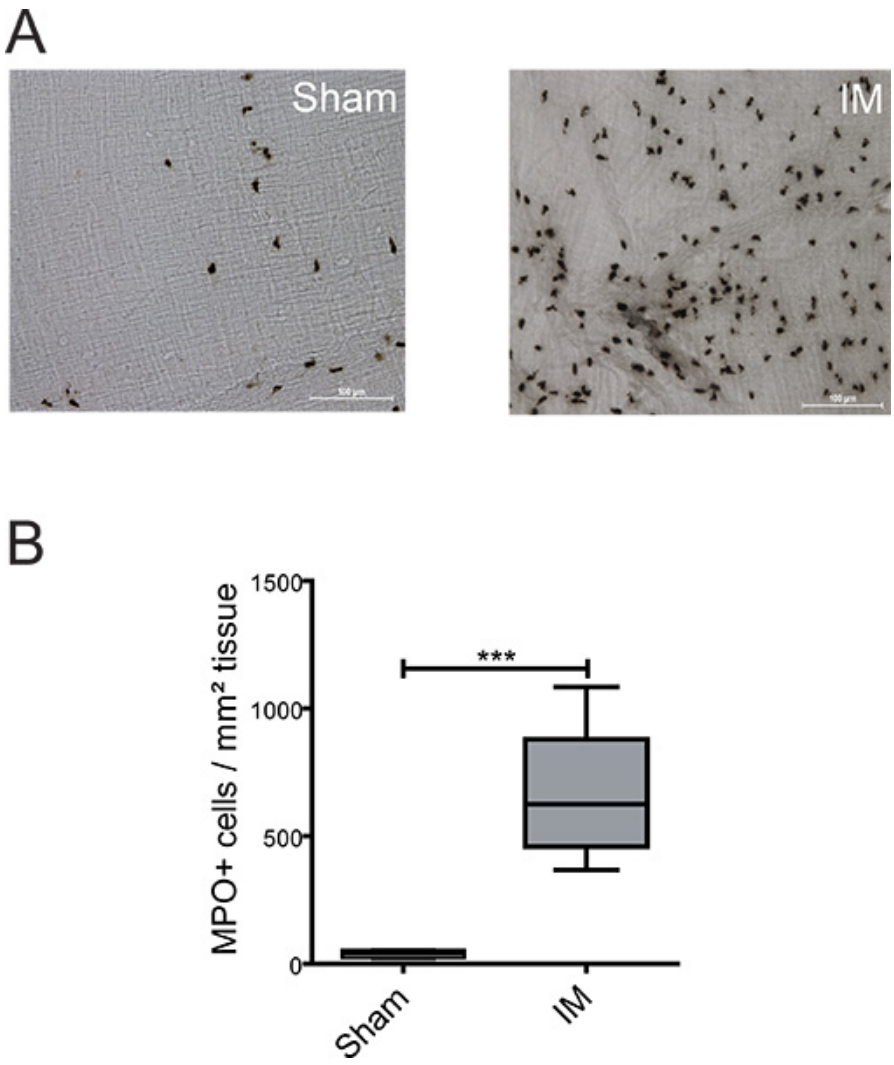

Figure 2. Detection of MPO positive cells within small bowel ME.(A) ME whole mounts were stained with Hanker Yates reagent for detection of MPO positive PMN 24 hr after laparotomy (sham) or IM procedure. (B) Quantification of MPO positive cell within 5 randomly chosen fields per mouse. n= 6 animals per group. *** = p<0.001, Student's t-test.

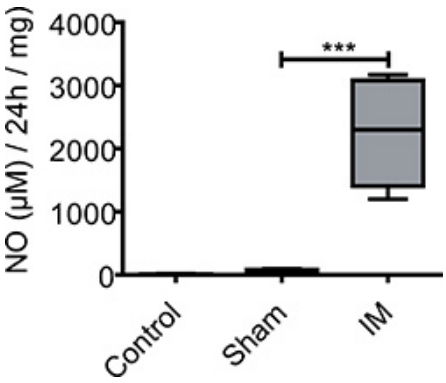

Figure 3. NO production in culture supernatants of ME. Muscle specimens from unoperated controls, sham operated and IM mice were taken 24 hr postoperatively and cultured for 24 hr. Untreated mice showed only a very low basal production of NO. After laparotomy NO liberation is increased without measuring significant differences between the control group and sham operated mice. 24 hr after IM NO production is dramatically increased compared with untreated or sham operated mice. n= 5 animals per group. *** = p<0.001, 1 way ANOVA.

Discussion

A comparative study analyzing different levels of IM (small bowel eventration for 10 min versus gently inspection of the small bowel using two cotton applicators versus IM with evacuation of small bowel content into the cecum) revealed a correlation between the extent of surgical manipulation and POI. Compared with the other groups IM showed the highest amount of leukocytes in the ME (PNMs, macrophages and mast cells) resulting in prolonged and severe POI.[8].

When establishing the model of IM, standardized operation and strength of bowel manipulation are critical steps. Touching the mesentery during this procedure must be strictly avoided to prevent hemorrhages. Insufficient strength during IM will result in a lack of small bowel distension. IM technique and strength varies between different surgeons can results in significant variation of POI occurrence. The same operator should manipulate all animals in one experiment.

Analyzing GIT and colonic transit *in vivo* is critical and must be performed carefully. Each animal should be housed during the procedure in darkened cages and in a silent room. Animals should be housed at least 7 days after arrival in the animal facility to adept to the environment (food, water, rooms, light dark cycles, immunological conditions). Measurement of both, GIT and colonic transit, allows one also to distinguish between intestinal inflammation and paralysis in treated and non-treated areas.[9] Another sophisticated method of *in vivo* motility recordings in awake rodents was described by Konigsrainer *et al.* [10] using strain gauge transducers placed on stomach, jejunum and colon. The great advantage of this method is that motility disturbances can be examined independently and continuously in different parts of the gastrointestinal tract in the same animal on consecutive days. However the production of strain gauge transducers and the necessary operation to implant them are elaborate compared to the GIT.

In POI, the dissemination of intestinal paralysis is known as gastrointestinal field effect.[11] A previous work of our group shows by the use of the present methods that immunocytes from intestinally manipulated small bowel disseminate to the unmanipulated colon.[12]

The surgical procedure should not result in any death or major complication (like hemorrhage or peritonitis). Measurement of GIT and PMN infiltrates in ME whole mounts demonstrates that IM is a highly reproducible and feasible model to induce POI in rodents.

Fixation and myeloperoxidase staining must be performed immediately after organ harvesting. Longer storage of the samples in aqueous buffer results in elution of myeloperoxidase from the tissue.

Measurement of nitric oxide is one example of mediator release from organ cultures: As shown before intestinal manipulation resulted in an upregulation of iNOS in macrophages of the ME leading to an excessive liberation of NO and intestinal dysmotility.[5] By measuring the concentration of NO in the supernatant of cultured ME a statement about the inflammatory extent or the efficacy of therapeutics is possible. Several other molecules (Il-6, TNF-alpha etc) can be easily examined in the supernatants of cultured tissue by classical ELISA or multiplex approaches like Luminex Multiplex Immunoassay[13] (Invitrogen, Carlsbad, CA, USA).

In summary, IM is a reliable and valuable model inducing bowel inflammation and subsequent motility disturbances. The simple mechanical separation of ME and tunica mucosa allows researchers to distinguish between the effect in both bowel wall compartments, that is of major interest since the intestinal ME has been shown to be a highly immunological active tissue. *In vivo* analysis of GIT and colonic transit has been developed as gold standards in detection and quantification of intestinal motility disturbances.

Disclosures

No conflicts of interest declared.

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