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Important Endpoints and Proliferative Markers to Assess Small Intestinal Injury and Adaptation using a Mouse Model of Chemotherapy-Induced Mucositis

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Cover Letter

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Xiaoyan Cao, Ph.D.
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Dear Dr. Cao

Thank you for allowing us to re-submit a revised version of the manuscript, "Important Endpoints and Proliferative Markers to Assess Small Intestinal Injury and Adaptation Using a Mouse Model of Chemotherapy-induced Mucositis". Every editorial comment has been reviewed and the suggested necessary changes in the manuscript have been done. Reviewer's comments have also been reviewed and separate responses to each comment have been provided, please see the rebuttal letter for specifications.

The selection of the crypt and villus for measurement of cell proliferation has been carefully revised and the manuscript has been added a supplementary figure to illustrate how this selection process is done. Additionally, the revised version of this manuscript considers both the advantages and limitations of using small intestinal wet weight as an endpoint for measuring enterocyte mass, compared to measuring citrulline.

Reviewer #1 kindly suggest that the discussing of previous models of 5-FU induced mucositis is shorted, since it is beyond the scope of the manuscript, while reviewer #4 suggested a better justification of this dose used. Although a better justification of the dose is desirable, we choose to meet the suggestion of reviewer #1 and remove the discussion of previous models of 5-FU induced mucositis. A better justification of the model is provided in the rebuttal letter.

The method and manuscript have not been published and is not under consideration for publication elsewhere.

Thank you for your consideration.

Sincerely,

H. Kissow

Hannelouise Kissow, MD, PhD

TITLE:

Important Endpoints and Proliferative Markers to Assess Small Intestinal Injury and Adaptation using a Mouse Model of Chemotherapy-Induced Mucositis

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KEYWORDS:

small intestinal injury, proliferative markers, BrdU, crypt depth, villus height, compensatory hyperproliferation

SUMMARY:

Here, we present a protocol to establish important endpoints and proliferative markers of small intestinal injury and compensatory hyperproliferation using a model of chemotherapy-induced mucositis. We demonstrate the detection of proliferating cells using a cell cycle specific marker and using small intestinal weight, crypt depth, and villus height as endpoints.

ABSTRACT:

Intestinal adaptation is the natural compensatory mechanism that occurs when the bowel is lost due to trauma. The adaptive responses, such as crypt cell proliferation and increased nutrient absorption, are critical in recovery, yet poorly understood. Understanding the molecular mechanism behind the adaptive responses is crucial to facilitate the identification of nutrients or drugs to enhance adaptation. Different approaches and models have been described throughout the literature, but a detailed descriptive way to essentially perform the procedures is needed to obtain reproducible data. Here, we describe a method to estimate important endpoints and proliferative markers of small intestinal injury and compensatory hyperproliferation using a model of chemotherapy-induced mucositis in mice. We demonstrate the detection of proliferating cells using a cell cycle specific marker, as well as using small intestinal weight, crypt depth, and villus height as endpoints. Some of the critical steps within the described method are the removal and weighing of the small intestine and the rather complex software system suggested for the measurement of this technique. These methods have the advantages that they are not time-consuming, and that they are cost-effective and easy to carry out and measure.

INTRODUCTION:

Intestinal adaptation is the natural compensatory mechanism that occurs when the bowel is lost due to disease or surgery[1,2]. After trauma, the gut undergoes a morphometric and functional adaptive response, characterized by crypt cell proliferation and increased nutrient absorption[3]. This step is critical in recovery, yet poorly understood. Experimental studies of the intestinal adaptive response have focused on the changes occurring after small bowel resection in mice, rats, and pigs, but understanding the molecular mechanism behind the adaptive response in other kinds of injuries (e.g., chemical or bacterial) is crucial to facilitate the identification of nutrients or drugs to enhance adaptation. Experimentally, different approaches have been used to describe the complex molecular and cellular index of small intestinal pathology, including histopathological scoring and measuring the outcome of injury. Despite this, what is absent from the literature is a detailed description of how to perform the procedures that are needed to obtain reproducible data. When identifying factors involved in adaptation, such as gut hormones, an easy, low cost, and reproducible animal model is warranted and here we suggest using a model of chemotherapy-induced intestinal mucositis (CIM).

One of the simplest and very informative endpoints of both injury and adaptation is to measure the mass of the small intestine (SI). We know that a hallmark of mucositis is apoptosis of enterocytes, time-dependent villus atrophy and reduced mitosis. Therefore, examining intestinal morphology is highly relevant in preclinical models[4,5]. In humans, a decline in plasma citrulline, a marker of functioning enterocytes, correlates with toxicity scores and inflammatory markers[6] in addition to the absorptive capacity[7], suggesting this amino acid is an excellent biomarker of mucositis. Citrulline can be measured in both mice and rats, and has shown excellent correlations with villus length[8], crypt survival[9], and radiation-induced mucositis[10].

A major advantage of measuring plasma citrulline is the ability to collect repeated measurements from one animal. However, multiple blood sampling in mice is restricted to a total blood volume of 6 µL/g/week and requires general anaesthesia. This unfortunately also limits the use of citrulline measurements in mice. Furthermore, the measurement of citrulline requires high-performance liquid chromatography[11,12], which is costly and time-consuming. Recently, we showed that citrulline levels in mice correlate significantly with SI weight ($p < 0.001$) (unpublished data), making citrulline a direct measurement reflecting enterocyte mass. A limitation to the measurement of SI weight is the necessity for the mice to be sacrificed and thus no repeated measurements within the same mouse are possible. Still the method provides the possibility to perform a variety of other tissue analyses directed to the research question, and these facts can conceivably make up for the additional use of animals. We, therefore, suggest using SI weight as an easy, low-cost, and fast biomarker of injury and adaptation in mice. To ensure reproducibility and acceptable analytic variation, the intestines should be carefully removed from the animal, flushed with saline, emptied and dried before weighing. In this article, we show exactly how this procedure is performed.

Another hallmark of mucositis is the loss of the proliferating cells in the crypts and a compensatory hyperproliferation during the regenerative period[3]. The cellular marker Ki67 has

been frequently used to determine fast proliferative cells by means of immunohistochemistry[13]. Even though Ki67 is a simple marker of proliferation, it has a tendency for imprecision as Ki67 is present during all active phases of the cell cycle (G1, S, G2, and M)[14]. Specific labelling is essential to detect replicating cells, which is why we suggest in situ incorporation of 5-bromo-2'-deoxyuridine (BrdU), a synthetic analogue of thymidine, as it is largely restricted to replicating cells in the S-phase[15]. BrdU is injected in the animals 150 minutes before sacrificing and cells can be subsequently detected with immunohistochemistry using BrdU specific antibodies. In this method article, we show exactly how to measure the area of BrdU immunopositive cells within a crypt using a free image software.

Morphologic and functional changes are often studied in 5-FU induced mucositis models, where the intestinal adaptation is assessed by villus height and crypt depth. During this study, we found that during the acute phase of mucositis, which is equal to the injury phase, proliferation measured by BrdU incorporation is not correlated with crypt depth. In contrast to this, crypt depth is significantly correlated with proliferation seen in the repair phase of mucositis, 3 to 5 days after induction. This suggests that the acute phase of mucositis is not measurable by crypt depth alone. We suggest that when using proliferation as an endpoint in the acute phase of mucositis mice, BrdU incorporation should preferably be used but when quantitating hyperproliferation in the later stage during the regenerative phase, crypt depth is a reasonable alternative to BrdU incorporation. The goal of this study was to describe this model in a way that it can be used by all researchers, both in the field of oncology but especially researchers not familiar with intestinal injury models.

The described model can be used to phenotype transgenic models according to the adaptive response using body weight, SI weight and crypt depth as endpoints. As an example, we show here how we used the model of 5-fluorouracil (5-FU) induced mucositis in a cellular knock out model with insufficient L-cell secretion[16]. Glucagon-like peptide-1 (GLP-1) and glucagon-like peptide-2 (GLP-2) are intestinal hormones co-secreted from the enteroendocrine L-cells in response to food intake[17,18]. GLP-2 is recognized as an important factor for intestinal healing, the regulation of mucosal apoptosis and the improvement of the barrier function of the SI[19-22]. Based on the literature, we hypothesized that endogenous hormones are essential for compensatory hyperproliferation occurring in the adaptive response after injury.

PROTOCOL:

All methods described were conducted in accordance with the guidelines of Danish legislation governing animal experimentation (1987). Studies were performed with the permission from the Danish Animal Experiments Inspectorate (2013-15-2934-00833) and the local ethical committee.

NOTE: Female C57BL/6J mice (~20−25 g) were obtained and housed eight per cage in standard 12 h light, 12 h dark cycle with free access to water and standard chow. Animals were left to acclimatize for one week before experiments began.

1. Induction of mucositis using 5-fluorouracil

1.1. Obtain 5-fluorouracil (5-FU) in a 50 mg/mL solution.

1.2. Weigh and record the body weight of the mice. From the body weight, calculate the amount of 5-FU for injection (e.g., 400 mg/kg).

1.3. Prepare a 1 mL syringe connected to a 27 G x 30 mm needle and fill the syringe with the calculated amount of 5-FU for injection.

1.4. Restrain the mouse by the scruff method. To do this, grab the base of the tail with one hand and place it on a toe-gripping surface, such as a wire bar lid. While positioning the tail with one hand, hold the scruff of the neck with the other hand.

1.5. Firmly position the body of the mouse across one hand by extending the forefinger and thumb back as far as possible. Place the tail between the fingers of this same hand to secure the mouse.

1.6. While maintaining the mouse in a firm but gentle grip, expose the ventral side of the mouse and insert the needle into the intraperitoneal cavity on the lower right or left quadrant of the abdomen. Aspirate to ensure proper placement and inject the 400 mg/kg 5-FU.

2. Tissue collection

2.1. Anesthetize the mouse with an intraperitoneal injection of ketamine/xylazine (100/10 mg/kg) diluted in saline solution (0.9% NaCl). Monitor the depth of anaesthesia by observing the pinch withdrawal reflex.

NOTE: The anaesthesia is a non-recovery anaesthesia.

2.2. Record the weight of the anesthetized mouse.

2.3. Place the mouse in a supine position and perform a laparotomy to expose the abdominal cavity, followed by an incision of the chest cavity to introduce pneumothorax.

2.4. Using scissors, sacrifice the animal by cutting open the diaphragm.

2.5. Remove the small intestine. Cut superior to the pylorus, carefully retract the small intestine away from the carcass until the cecum is reached and make a cut just before the cecum.

2.6. Using forceps, gently clamp the proximal lumen shut. Using a 1 mL syringe with a 25 G needle attached, flush the small intestine with saline to remove the feces.

2.7. Place the small intestine on clean blotting paper to carefully remove excess saline.

2.8. Record the weight of the flushed tissue.

2.9. Calculate the flushed small intestinal weight as a percentage of body weight.

3. Small intestine histology

3.1. **Tissue preparation**

3.1.1. In a fume hood, use scissors to cut 3 cm sections of the flushed intestinal tissue from each mouse from the duodenum, jejunum and ileum and fixate sections in 10% formalin for 24 h at room temperature.

NOTE: The fixative volume should be 5−10 times of the tissue volume.

3.1.2. Transfer the fixed tissue to a cutting board.

3.1.3. Using a scalpel, trim the tissue to approximately 1 cm and transfer into an embedding cassette.

NOTE: The cassette should be labelled with the sample identification (ID) in pencil.

3.1.4. Submerge the cassette in 70% ethanol and store at 4 °C until further processing.

3.2. **Tissue embedding in paraffin**

3.2.1. Dehydrate the tissue by placing in ascending alcohol solutions. Place the cassette in 70% ethanol for 1 h, followed by 80% ethanol for 1 h, 95% ethanol for 1 h and 100% ethanol for 1.5 h. Transfer the cassette from 100% ethanol to xylene for 1.5 h.

3.2.2. Immerse the cassette with paraffin wax heated between 58−60 °C. Use forceps to position the tissue in a transverse orientation and leave the blocks to cool more than 24 h until solid.

3.3. **Cutting tissue using a microtome**

3.3.1. Place block, tissue face down on ice for 5 min. Using a microtome, trim the paraffin blocks at a thickness of 10−30 µm to expose the tissue surface.

3.3.2. Cut cross-sections of the tissue block between 3−5 µm, making transverse sections from the duodenum, the jejunum and the ileum.

3.3.3. Place paraffin ribbon in a water bath set between 40−45 °C. Use forceps to separate the sections.

3.3.4. Use microscope slides to pick up the sections from the water.

3.3.5. Air dry the slides for 30 min before staining.

NOTE: For storage over a longer period of time, store slides in a fridge.

3.4. **Hematoxylin-eosin staining of tissue**

3.4.1. Place the microscope slides in a histology cradle. Deparaffinize the slides in a heating cabinet set at 60 °C for 60 min.

NOTE: Slides directly from the fridge should be left to reach room temperature before placing them in a heating cabinet.

3.4.2. In the fume hood, rehydrate the tissue in 3 changes of a clearing agent (**Table of Materials**), make 20 dips in the first clearing agent jar and then let them stand for 7−8 min in each of the two additional clearing agent jars. Transfer the slides to descending alcohol solutions, start with 3 changes of 99% ethanol, followed by 2 changes of 96% ethanol and 70% ethanol. Make a minimum of 20 dips in each jar. Transfer to running water and let the slides stand for 5 min.

3.4.3. Immerse the slides in filtered Meyer's hematoxylin for 1 min.

3.4.4. Wash the samples in running tap water for 5 min.

3.4.5. Immerse sections in the eosin stain for 1−2 min, then rinse in tap water.

3.4.6. Dehydrate the tissue by placing in ascending alcohol solutions. Start with 70% ethanol, followed by 96% ethanol, 96% ethanol, and 4 times 99% ethanol. Make a minimum of 20 dips in each jar, and let the cradle stand in 99% ethanol until they are mounted.

3.4.7. Mount coverslips by applying a small amount of the mounting medium to the surface of the slides. Put the coverslip on top of the mounting medium without creating bubbles under the coverslip. Let it dry for 24 h.

3.4.8. Examine the tissue using a light microscope connected to a camera to obtain histological photos. Use a 5x objective to take snapshots until a full coverage of the tissue slide is reached.

4. Measurement of crypt depth and/or villus height

4.1. Download and install the analysis software (i.e., Zen Lite, **Table of Materials**).

4.2. Open the image in the software and connect to the camera. Take snapshots in camera mode with 40x objective.

4.3. In processing mode, open the snapshot.

4.4. To measure the crypt depth: in the **2D view**, select the line tool from the graphics tab. Choose a well-orientated crypt and start the line at the bottom of a villus and finish at the bottom of the crypt. Repeat this action for 20 well-orientated crypts (**Supplementary Figure 1**).

4.5. To measure the villus height: in the **2D view**, select the line tool from the graphics tab. Choose a well-orientated villus and start the line at the end of the luminal projection and finish at the start of the crypt. Repeat this action for 20 well-orientated villi (**Supplementary Figure 1**).

4.6. Switch from **2D view** to the **Measure view** to display the measurements.

4.7. Export the measurements and calculate the average of the crypt depth and villus height.

5. BrdU quantification (proliferation) by immunohistochemistry

5.1. **Injection of the BrdU solution**

5.1.1. Weigh and record the body weight of the mice.

5.1.2. In a fume hood, prepare a representative amount of 0.5% w/v bromodeoxyuridine (BrdU) solution in phosphate buffered saline (PBS).

5.1.3. In the fume hood, inject 50 mg/kg of BrdU by intraperitoneal injection.

NOTE: To ensure that each mouse is euthanized at exactly 150 min post BrdU injection, inject each individual mouse in succession with 10−20 min interval in between in order to properly perform the tissue collection.

5.1.4. Record the time of injection.

5.1.5. Wait 150 min, then anesthetize mice and collect the tissue as described in steps 2.1−2.7. Perform BrdU immunohistochemistry as described in section 5.2.

5.2. **BrdU immunohistochemistry**

5.2.1. Prepare the tissue as described in steps 3.1.1−3.3.4.

5.2.2. For antigen retrieval, place a slide cradle with the sections in a glass histology jar and fill it with the EDTA buffer (pH 9) in a microwave oven for 1 min at 750 W followed by 9 min at 350 W. Repeat cycle.

NOTE: Ensure that the tissue does not dry out, which might require adding more buffer to the jars between heating.

5.2.3. Wash sections 2−3 times in tris-buffered saline and polysorbate 20 (TBS-T) buffer for 3 min each.

5.2.4. Apply 5 drops of peroxide block (**Table of Materials**) for 10−15 min at room temperature to block endogenous peroxidase activity. Wash sections 2−3 times in TBS-T buffer for 3 min each.

5.2.5. Apply 5 drops of rodent block buffer (**Table of Materials**) for 30 min at room temperature, to block non-specific binding. Wash sections 2−3 times in TBS-T buffer for 3 min each.

5.2.6. Incubate the sections with a monoclonal mouse anti-BrdU antibody diluted 1:500 for 1 h at room temperature. Wash sections 2−3 times in TBS-T buffer for 3 min each.

5.2.7. Visualize immunopositivity using 3,3'-diaminobenzidine (DAB) by applying 5 drops of horseradish peroxidase to each slide and incubate for 15 min at room temperature. Transfer the sections to a fume hood, add 150 µL of DAB and incubate for 5 min.

NOTE: Always wear gloves when handling DAB and dispose of waste appropriately.

5.2.8. Rinse sections in deionized water.

5.2.9. Dehydrate the tissue and mount a coverslip as described in steps 3.4.6 and 3.4.7.

5.3. **Visualization of the immunoreactions**

5.3.1. Visualize the tissue sample with a light microscope connected to a camera.

5.3.2. Use the analysis software to obtain microscope images using a 20x objective of all sections and save files in the .JPG format.

5.3.3. Take a snapshot of a stage micrometer using a 20x objective, which will be used as a calibration tool in ImageJ software.

5.4. **Measuring the area of BrdU immunoreactive cells**

5.4.1. Install ImageJ software (**Table of Materials**).

5.4.2. Assigning scale to an image in ImageJ: Open the stage micrometer image using the ImageJ **File | Open** menu command. Select the straight-line selection tool.

5.4.3. Select the straight tool and draw a straight line on the micrometer image to define a known distance.

5.4.4. Select **Set Scale** under the **Analyse** menu.

5.4.5. Enter a value in the **Known Distance** box and define the unit of length in the **Unit of Length** box. Select **Global** so that this calibration applies to all images that are opened in this ImageJ session.

5.4.6. Open an image of interest by using the ImageJ **File | Open** menu command to measure the area of the BrdU immunoreactive cells per crypt.

5.4.7. Set the color graphics to 8-bit under the **Image/Type** menu.

5.4.8. Increase the contrast of the image under **Process/Enhance contrast** and set the saturated pixels to 0.4%.

5.4.9. Apply a threshold to the image to segment the immunopositivity. Select **Image/Adjust** under menu, then **threshold** and select the color **Red** (dark area shown in red). Choose a threshold of around 10−20% by moving the threshold bar.

NOTE: Choose a threshold that includes all the BrdU positive cells with as little background as possible. The chosen percentage should apply to all sections.

5.4.10. Choose a well-orientated crypt, i.e., a complete crypt from intact tissue, and select the free hand selection tool to mark the area around it.

5.4.11. To measure the threshold area, select the **Analyse** tab, followed by **Analyse particles**. In the **Analyse particle** window, set **Size** to **20-infinity**, click the box **pixel units**, set **Circularity** to **0.00−1.00**, and set **Show** to **Outlines**. Click boxes **Display Results**, **Summarize**, and **Include holes**.

NOTE: The values for BrdU positive cells are automatically generated in a result window, showing the individual measurements for each BrdU positive cell. Furthermore, a summary window is automatically generated, showing the number of counts, total area, average size and percentage of area with BrdU positive cells.

5.4.12. Repeat steps 5.4.10 and 5.4.11 to measure a new crypt. The results of all measured crypts will be displayed in the **Summary** window.

REPRESENTATIVE RESULTS:

In the first experiment, we induced mucositis in mice at day 0 and sacrificed a group of mice each day for 5 consecutive days. When measuring the SI weight, we found that this parameter decreased from day 2 until day 4 suggesting a loss in the enterocyte mass. We also found that at day 5, the SI weight was not significantly different from day 0 (untreated mice) (**Figure 1**). The proliferation measured by the incorporation of BrdU was almost abolished at day 1 and day 2, but at day 4 and day 5 there were approximately four-fold and five-fold increases in proliferation, respectively (**Figure 2**). This hyperproliferation was also illustrated when measuring the crypt depth (**Figure 3**). What is not illustrated by measuring crypt depth is the loss in proliferating cells at day 1 and day 2, where crypt depth was reduced by approximately 13% but was not

significantly different from the healthy mice. We could show that, in the regenerative phase of mucositis there was a strong correlation between BrdU incorporation and crypt depth, but this did not count for the acute phase indicating that crypt depth as an endpoint might not be suitable in the acute phase of mucositis (**Figure 4**).

In the second study, we examined mucostis in our transgenic mouse model with insufficient L cell secretion. Mice with deficient GLP-1 and GLP-2 showed a severe loss of body weight (BW) and a decrease in SI weight in the recovery phase, which was highly significant compared to the wild type (WT) 5-FU mice ($p < 0.01$) (**Figure 5A,B**). Furthermore, the transgenic mice failed to show compensatory hyperproliferation; crypts were significantly shorter than in both WT mice and healthy controls. Contrary to this the WT mice showed an increase in crypt depth as a sign of hyperproliferation (**Figure 5C**).

FIGURE LEGENDS:

Figure 1: Small intestinal weight. Mice were sacrificed 1 to 5 days after 5-FU injection at day 0 and the intestinal weight was measured as described. Results are shown as mean ± standard error of the mean (SEM). n = 13. This figure has been modified from Hytting-Andreasen et al.[16].

Figure 2: BrdU immunopositive cells per crypt for the duodenum, jejunum, and ileum of the SI. Mice were sacrificed 1 to 5 days after 5-FU injection at day 0 and BrdU incorporation was quantified by immunohistochemistry as described. Results are shown as mean ± SEM. n = 13. *p < 0.05, ***p < 0.001 compared to day 0 (analysis of variance [ANOVA] followed by Dunnett multiple comparison test). ***p < 0.001 compared to day 0 (ANOVA followed by Dunnett multiple comparison test). This figure has been modified from Hytting-Andreasen et al.[16].

Figure 3: Measurement of crypt depth. Mice were sacrificed 1 to 5 days after 5-FU injection at day 0 and crypt depth was measured as described. Results are shown as mean ± SEM. n = 13. *p < 0.05, ***p < 0.001 compared to day 0 (ANOVA followed by Dunnett multiple comparison test). This figure has been modified from Hytting-Andreasen et al.[16].

Figure 4: Correlation of crypt depth and BrdU. Crypt depth (in duodenum, jejunum, and ileum) is correlated to BrdU incorporation at each day of sacrifice using two-tailed Pearson correlation test.

Figure 5: GLP-1 and GLP-2 deficient mice fail to regenerate after acute mucositis. (**A**) Change in BW (%), (**B**) SI weight (g), and (**C**) crypt depth in ileum (µm). Results are shown as mean ± SEM. Tg = transgenic mice; WT = wild type mice; 5-FU = 5-fluorouracil. n = 4–8. *p < 0.05, **p < 0.01, ***p < 0.001 compared to healthy control (WT saline), a = p < 0.05, aa = p < 0.01, aaa = p < 0.001 compared to WT 5-FU (two-way ANOVA followed by Bonferroni multiple comparison test [BW] or ANOVA followed by Dunnett multiple comparison test). This figure has been modified from Hytting-Andreasen et al.[16].

Supplementary Figure 1: Small intestinal tissue before the induction of mucositis, during the acute phase and the recovery phase of mucositis. (**A**) Haemotoxylin and eosin (H&E) staining and (**D**) BrdU staining in untreated mice. The black dotted line in panel A exemplifies a well-orientated crypt, whereas the dotted green line demonstrates a well-orientated villus. (**B**) H&E staining and (E) BrdU staining during the acute phase of mucositis. (**C**) H&E staining and (**F**) BrdU staining during the recovery phase after the induction of mucositis. Scale bar = 100 µm.

DISCUSSION:

Here, we demonstrate a widely accessible method to study SI injury and regeneration in a mouse model. A wide variety of preclinical animal models of intestinal injury exist, but it is vital we understand that each model is unique and that the endpoints must be appropriate to answer the research question. This model is excellent to study adaptive response to injury, but the endpoints should be modified when using the model as a pre-clinical model of mucositis. However, translation from animal models to patients is challenging[23]. Our suggested endpoints of SI weight and proliferation should be limited to the study of adaptive response only. The study of endogenous factors often requires the use of transgenic mice, and even if small bowel resection is possible in mice[1], this model could be an alternative to avoid post-operative mortality. When applying this model to transgenic mice, it is important to watch the mice carefully and monitor their weight every day. During this study, some of the mice experienced a weight loss of up to 30%, which is quite substantial. To avoid high mortality in sensitive phenotypes, we suggest performing pilot studies in transgenic mice, since dose adjusting might be necessary.

A critical step within the described method is the removal and weighing of the SI. It is important that the removal and handling be performed in the same manner and by the same researcher each time to avoid large inter-assay variations.

The consistency of crypt and villus selection is important to avoid variance and bias when measuring crypt depth and villus height. When embedding the tissue in paraffin, the intestines are positioned in an upright position to make transverse cuts, thus increasing the possibility for intact villi and crypts. After cutting of the tissue, a well-orientated crypt and/or villus is selected. Selection is based on the full visualization of the whole crypt and villus in the same plane and the presence of clear borders of cells within the crypt and villus. A limitation to this method is the somewhat subjective approach when selecting a well-orientated crypt since the selection of a well-orientated crypt and/or villi is made after cutting the tissue. A previous study[24] has presented an alternative method to overcome this limitation, where they use microdissection. In this method, villi and crypts are selected while observing under a microscope, prior to the tissue being cut, thus making it possible to ensure that an intact crypt and villi are being dissected from the tissue.

In contrast to previous methods used to quantity BrdU positive cells[25,26], this protocol describes the area of BrdU positive cells per crypt, which provides a fast way to quantitate proliferative cells within each crypt. This technique, however, may be somewhat restrictive since it requires a more profound knowledge of the software suggested for the measurement of this technique. A future application of this protocol could be to create a more automatic generated method to

quantify and measure the BrdU positive cells.

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DISCLOSURES:

The authors have nothing to disclose.

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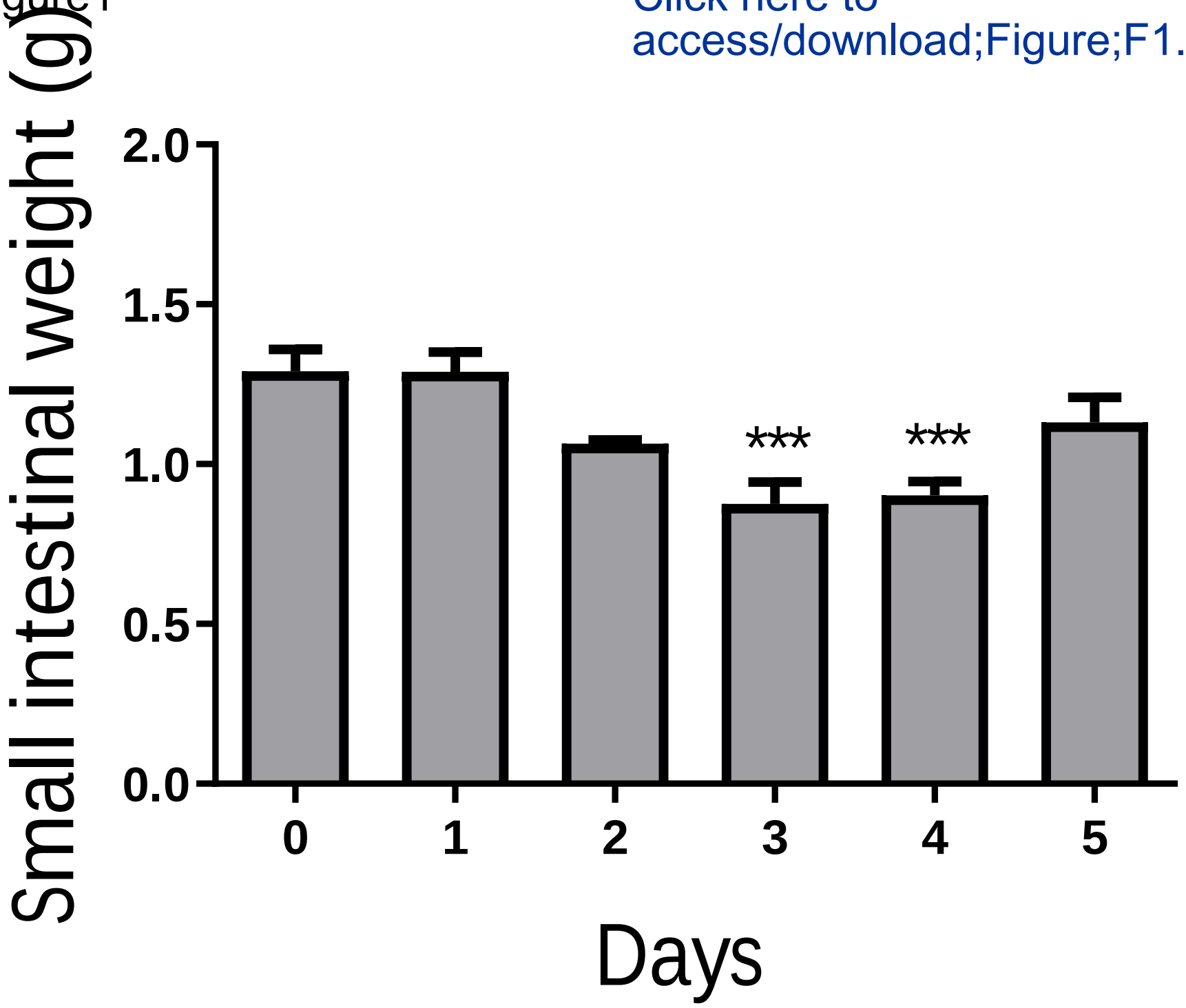

Figure2

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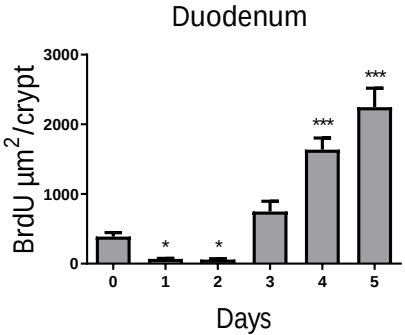

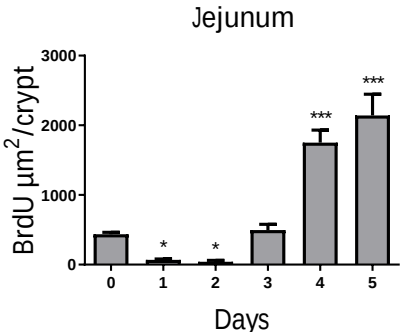

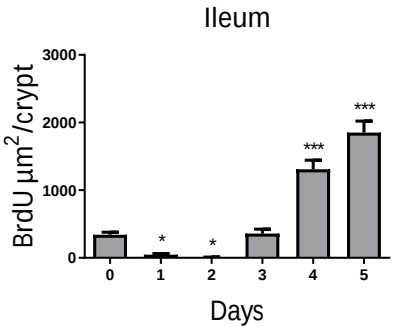

Figure3

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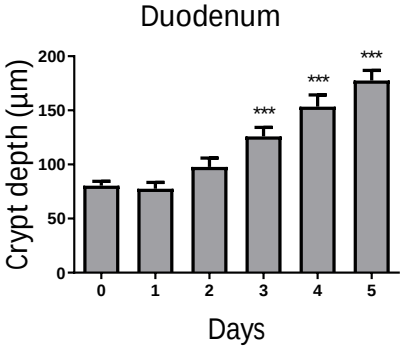

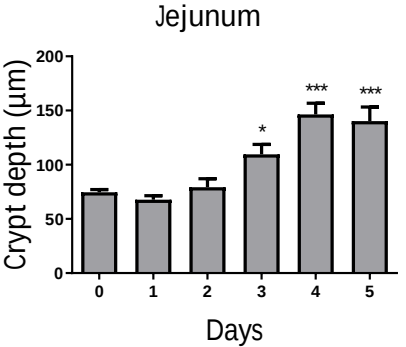

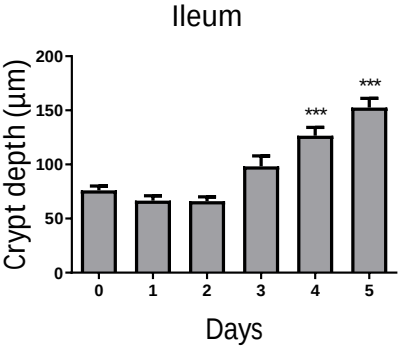

Figure4

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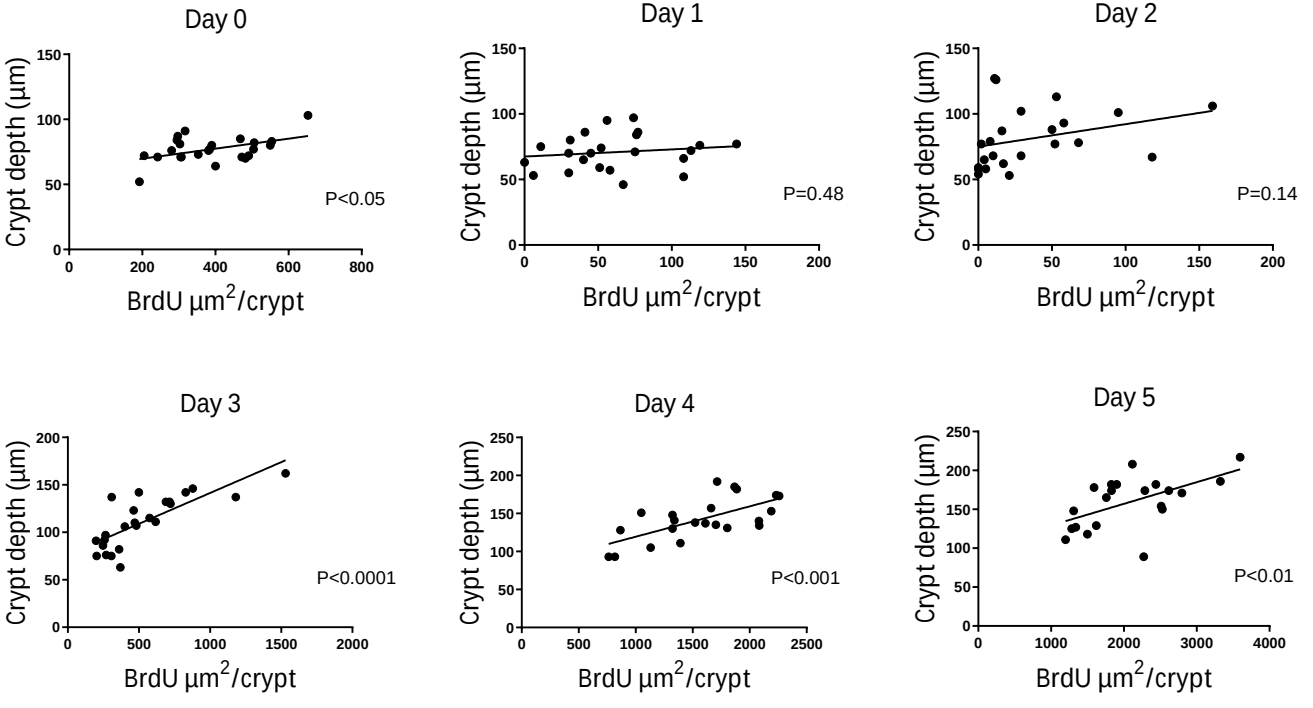

Figure5

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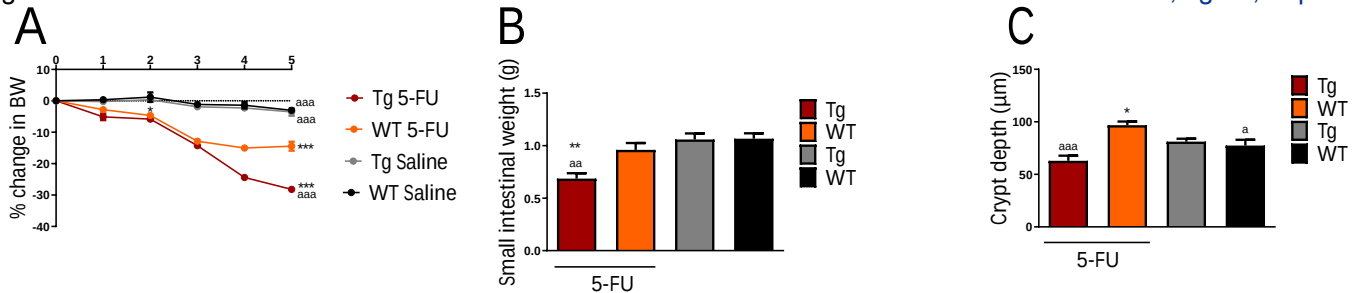

Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
5-Fluorouracil	Hospira Nordic AB, Sweden	137853	
BrdU	Sigma-Aldrich, Germany.	B5002	
d-limonene - Histoclear II (Clearing agent)	National Diagnostics, USA	HS-202	
DAB Quanto Chromogen	DAB Substrate Kit, Thermofisher Scientific, Denmark	TA-125-QHDX	
DAB Quanto Substrate	Thermofisher Scientific,	TA-125-QHDX	
Eosin Y	Sigma-Aldrich,	230251	
Ethanol ≥70% (v/v), TechniSolv	VWR chemicals, Denmark	83,801,360	
Ethanol ≥96% (v/v), TechniSolv	VWR chemicals, Denmark	83,804,360	
Ethanol absolut ≥99.5%, TechniSolv	VWR chemicals, Denmark	83,813,360	
Formalin solution, neutral buffered, 10	Cell Path Ltd, Powys,	BAF-5000-08A	
Hematoxylin Solution, Mayer's	Sigma-Aldrich,	MHS16	
Horseradish peroxidase	Ultravision Quanto	TL-060-QHDM	
ImageJ Software	LOCI, University of Wisconsin		https://imagej.nih.gov/ij/
KetaminolVet	Merck, New Jersey,	511485	
Lab Vision Antibody Diluent OP	Thermofisher Scientific,	TA-125-ADQ	
Monoclonal mouse anti-BrdU antibody	Thermofisher Scientific, Denmark.	MA1-81890	
Parafin wax	Sigma-Aldrich, Germany.	327204	
Peroxide Block	Ultravision Quanto	TL-060-QHDM	
Pertex	HistoLab, Sweden	840	
Rodent Block buffer	Ultravision Quanto	TL-060-QHDM	
RompunVet Xylazine	Rompunvet, Bayer,	148999	
Tris/EDTA pH 9 buffer	Thermofisher scientific,	TA-125-PM4X	

Xylene	Sigma-Aldrich,	214736	
Zen Lite Software (Blue edition)	Carl Zeiss A/S		oscopy/int/products/micros

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Author(s): Anna Billeschou, Jenna Elizabeth Hunt, Hannelouise Kissow

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Editorial comments:
Changes to be made by the author(s) regarding the manuscript (59236_R1_clean):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

The manuscript has been revised and undergone proofreading.

2. Please revise lines 114-117, 391-394 to avoid previously published text.

The lines have been revised.

3. Keywords: Please consider removing Zen Light software and ImageJ software from the list.

After revising, we have now removed Zen Light software and ImageJ software from the keywords list.

4. In-text citations: Please remove the square brackets enclosing the reference numbers. The corresponding number from the reference list mast appear superscripted without a space after the word/group of words it applies to but before any punctuation.

All square brackets enclosing the reference number has been removed and does now appear without space after the word/group of words it applies.

5. Please abbreviate liters to L (L, mL, µL) to avoid confusion.

Liters have now all been abbreviated to L

6. Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.

A space is now added between all numerical values and their corresponding units.

7. 2.4: How large is the incision?

The text has been edited to ‘cut’ instead of ‘incision’ since the intestines are being separated from the ventricle and cecum.

8. 4.2: Please specify the image used (from step 3.4.8?). How many snapshots are taken?

On the microscope, the objective used to look at the tissue section should preferably be an x5 or x2.5 so that as few pictures are taken to cover the whole tissue section.

The number of snapshots that is needed should be until the fully cover the tissue on the slides is reached

9. Please replace commercial language (HistoClear) with a generic term.

The commercial name HistoClear have been replaced with a generic term and is referred to as a clearing agent in the protocol.

10. Figure 2: Please describe the left, middle and right panels in the figure legend.

A more detailed figure heading has been added to the figure legend, describing that figure 2 contains an illustration of BrdU immunopositive cells per crypt for all the three parts of the SI, the duodenum, jejunum, and ileum.

11. Figure 4: Please apply superscript formatting to the number "2" in the unit of "µm2".

Superscript formatting is now added.

12. Reprinted figures must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

This figure has been modified from Hytting-Andreasen et al. and a citation is added to the figures.

13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Please do not abbreviate journal titles. See the example below:
Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

A JoVe reference style has now been added to the references.

14. Table of Materials: Please ensure that it has information on all relevant supplies, reagents, equipment and software used, especially those mentioned in the Protocol.

The table of materials have been updated and has information on all relevant supplies, reagents, equipment, and software used in the protocol.

Reviewers' comments:

Please note that the reviewers raised some significant concerns regarding your method and your manuscript. Please revise the manuscript to thoroughly address these concerns. Additionally, please describe the changes that have been made or provide explanations if the comment is not addressed in a rebuttal letter. We may send the revised manuscript and the rebuttal letter back to peer review.

Reviewer #1:

Manuscript Summary:

The authors have made a significant effort to improve the manuscript, with the introduction now providing a much better rationale for the described work. I still question the novelty of the work described, with significant detail being used to describe fairly traditional and robust methods (e.g. H&E). Despite this, I do recognise the need to create universal methods for assessing intestinal injury, particularly in mucositis research in which studies are challenging to compare due to variations in endpoint analyses.

Thank you again for reviewing this manuscript. We do agree that traditional and robust methods (e.g. HE staining, which most researchers are familiar of) are described in details, but this is due to journal requirements. The methods that we aim to describe and film are not novel, but still not trivial.

Major Concerns:

The authors describe that plasma citrulline requires highly specialised methods and as such, small intestinal wet weights provide a cheaper and more simple method of analysing enterocyte mass. Whilst this is true, the authors have neglected to acknowledge that small intestinal weights require the animal to be sacrificed and prevent the ability to collected repeated measures from one animal. As such, it could be argued that small intestinal wet weights is an unethical alternative to plasma citrulline analysis. Furthermore, the costs of additional animals and associated husbandry must be considered in the context of intestinal wet weights as an endpoint. This needs to be discussed / acknowledged.

Thank you for pointing this out. We do acknowledge citrulline as a very good and strong method for measurement of enterocyte mass and in this manuscript we simply present an alternative method for measuring enterocyte mass. In the manuscript, we have now considered some of these points and discussed these further.

Added to the manuscript;

A major advantage of measuring plasma citrulline is the ability to collect repeated measurements from one animal. However, multiple blood sampling in mice is restricted to a total blood volume of 6µL/g/week and requires general anaesthesia. This unfortunately also limits the use of citrulline measurements in mice. Further, measurement of citrulline requires high-performance liquid chromatography, which is costly and time consuming. Recently, we showed that citrulline levels in mice correlate significantly with SI weight ($p<0.001$) (unpublished data), making citrulline a direct measurement reflecting enterocyte mass. A limitation to the measurement of small intestinal weight is the necessity for the mice to be sacrificed and thus no repeated measurements within the same

mouse is possible. Still the method provides the possibility to perform a variety of other tissue analysis directed to the research question, and these facts can conceivably make up for the additional use in animals. We therefore suggest using SI weight as an easy, cheap and fast biomarker of injury and adaptation in mice. To ensure reproducibility and acceptable analytic variation, the intestines should be carefully removed from the animal, flushed with saline, emptied and dried before weighing. In this report, we show exactly how this procedure is performed.

The authors spend excessive time discussing previous models of MTX induced mucositis, and the dosing schedules used. Whilst this is of interest, this needs to be shorted as it is beyond the scope of the manuscript. Your study is primary focused on endpoints of intestine injury, not the development of an MTX mucositis model. Perhaps consider discussing other primary endpoints used by other studies, not the dosing strategies.

Thank you for pointing this matter out. We do spend some time justifying why we used 400 mg/kg 5-FU in mice as the dose in our model. We agree that this section is a bit redundant and we have therefore deleted this section since it is beyond the scope of the manuscript.

You mention that plasma citrulline and SI weight correlate in your model (unpublished data). Is it possible to include this in your paper? This would further validate the importance/rigor of small intestinal weights as an alternative to plasma citrulline.

Whilst this is a very good suggestion, we can unfortunately not include our data concerning correlation between plasma citrulline levels and SI weight. Our plan is to get this specific data published in the near future.

Whilst you acknowledge that an intact crypt is one that is whole, but significant bias and variation could be introduced by simply including obliquely cut crypts in your analysis. How do you ensure that the crypts you are including in your analysis are
1) intact, and 2) not oblique. Are there alternative methods of fixing and preparing the tissue that overcome this? Please provide greater detail on crypt selection (perhaps a representative image of what constitutes an intact crypt vs a non-intact crypt would be useful?), and please acknowledge the limitations of this method.

Thank you for pointing this out. We have now disuses this method and its limitations in the discussion of the manuscript.

Added to the Manuscript;

The consistency of crypt and villus selection is important to avoid variance and bias when measuring crypt depth and villus height. When embedding the tissue in paraffin, the intestines are positioned in an upright position to make transverse cuts, thus increasing the possibility for intact villus and crypts. After cutting of the tissue, a well-orientated crypt and/or villi are selected from a designated criteria set, where crypts and villus are represented from crypt bottom to the top of the villus, with clear borders of cells within the crypt and villus. A limitation to this method is the somewhat subjective approach when selecting a well-orientated crypt, since the selection of a well-orientated crypt and/or villi is made after cutting the tissue. A previous study[1] has presented an alternative method to overcome this, where they use microdissection. In this method, villus and crypts are selected while

looking under a microscope, prior to the tissue being cut. Thus, making it possible to ensure that an intact crypt and villi are being dissected from the tissue.

Minor Concerns:
Please be consistent with your spelling of diarrhea (vs diarrhoea).

Thank you for pointing this out, it is now corrected.

Please avoid using etc. in sentences.

This is now corrected.

Reviewer #3:
Manuscript Summary:
This study is well-organized. Intestinal mucositis is clinically important issue. Thus, this manuscript is useful for the basic researchers in this field.

Thank you for some very insightful arguments for this manuscript.

Major Concerns:
In general, intestinal mucositis is characterized by shortening of villi and crypt destruction. This reviewer highly recommends showing the plane results of villus hight and crypt destruction. Further, the typical histological images of intestinal mucositis with normal mucosa could be useful.

Thank you for these very important arguments. We have added pictures of villus and crypts within normal tissue as well as pictures from the different phases of mucositis. See supplementary figure 1.

Apoptosis is believed to be a critical event in the occurrence of intestinal mucositis induced by 5-FU. Does the authors have some results of apoptosis?

We, unfortunately, do not have any results of apoptosis within this experiment.

Inflammatory responses are also important events. So, the determination of various inflammatory parameters such as inflammatory cell infiltration and cytokine expressions could be useful. This reviewer is interested in the correlation between cell proliferation and these inflammatory responses after induction of intestinal mucositis.

The correlation between the injury-induced proliferation in the recovery phase is extremely interesting. Multiple studies have investigated the acute phase and the inflammatory response and injury/mucositis, but we are very eager to investigate the proliferative response and how this correlates to inflammation in the future.

Unfortunately, we do not have sample material from the described study to perform further analysis, but we do agree that would have been interesting.

Reviewer #4:

Manuscript Summary:

A method of histologic measurement of crypt depth which correlates with BrdU incorporation during the recovery phase in a mouse model of 5-FU acute injury and repair is presented.

Thank you very much for your helpful inputs regarding this manuscript. A comment has been attached to each valid point to explain the method used further.

Major Concerns:

1) There is no mention of how or when the mice are sacrificed, it is unclear whether small intestines are partially resected from the same live mice longitudinally, removing organs from live animals is ethically unacceptable.

We neglected to mention how and when the mice were sacrificed, thank you for pointing this very important detail out. It has now been described in the protocol.
The small intestines are not partially resected from the same live mice longitudinally. The SI for each mouse is removed after the mouse has been sacrificed and then cut into minor sections for histology and assay use.

2) 400mg/kg of 5FU is an excessive dose, definitely at the high end of doses reported in the literature, needs better justification, do smaller more physiological doses trigger intestinal changes measurable by the proposed methods? If not then the utility of the proposed methods is limited.

When studying 5-FU induced mucositis in mice, doses vary between 300-450 mg/kg in a single i.p. injection and the acute phase is often studied 3 days after injection[2-4]

Our aim is actually to use a dose that only triggers small changes that can be detected with our methods. We have substantial experience with the chosen dose in normal healthy mice and we have never seen diarrhea, pilo erection or bleeding and BW loss is usually 5-10% however, this depends heavily on the animal facility (noise etc). Therefore, we do not agree that this dose is an excessive dose, when only one dose is administered (it is of course different in models of multiple dosing regimens). However when using transgenic mice that is suspected to be more prone to mucositis we heavily encourage researchers to perform dose response experiments, especially from our own experience that showed that this dose was too high in mice with a cellular knock down of GLP-2

3) A weight loss of 30% with this high dose is ethically unacceptable, although the authors try to make a point about the use of transgenic mice/weight loss this point is not clearly articulated. The approved recommendation in animal protocols is to sacrifice animals when they reach 20% weight loss, which was not done.

In all our former experiments, there was no indication of such a massive weight loss for non-transgenic mice and thus the model duration over 5 days. We agree that a weight loss of 30% is absolutely unethical, but due to the fact that the severe weight loss came as a surprise, we did not sacrifice them before. From this experience we therefore advice dose-response experiments in mice with any genetic modifications.

4) The utility of the ZEN software for crypt depth measurements needs to be better explained, why can't the same measurements be done with Image J? This is probably the only interesting aspect of this work, but it is not sufficiently explained.

It is possible to use Image J instead of Zen software when measuring crypt depth. In this manuscript, we choose to use Zen software for the morphometric analysis since we found this software to be a bit more user friendly, but it is of cause a matter of opinion.
Zen software does not provide the features we used to measure area of BrdU staining and for this reason, we choose to use Image J, we therefore present the readers for two free software's.

5) The observation that crypt depth is not reduced after acute injury on days 1-2 needs to be explained.

The crypts are certainly damaged after chemotherapy, due to apoptosis of proliferating cells in the crypt. However, our point is that this is not reflected when only measuring the height of the crypts since a proportional large part of the height in this state is cells not undergoing apoptosis (e.g paneth cells). Therefore, the measurement of crypt height does not reflect proliferative activity. The crypts *are* reduced in height in jejunum and ileum (about 13% in ileum) showing that they are damaged, but due to a relative small sample size, this is not statistically significant.

Minor Concerns:
1. The correlations shown between BrdU and crypt depth over time need a better explanation in the context of injury and repair.

A better explanation in context to correlation between BrdU and crypt depth have been added to the protocol.

Morphologic and functional changes are often studied in 5-FU induced mucositis models, where the intestinal adaptation is assessed by villus height and crypt depth. During this study, we found that during the acute phase of mucositis, which is equant to the injury phase, proliferation measured by BrdU incorporation is not correlated to crypt depth. In contrary to this, crypt depth is significantly correlated to proliferation seen in the repair phase of mucositis, 3 to 5 days after induction. This suggests that the acute phase of mucositis is not measurable in crypt depth alone.

We suggest that when using proliferation as an endpoint in the acute phase of mucositis mice BrdU incorporation should preferably be used but when quantitating hyperproliferation in the later stage during the regenerative phase, crypt depth is a reasonable alternative. The goal with this study was to describe this model in a way that it can be used for all researchers, both in the field of oncology but especially researchers not familiar with models if intestinal injury.

2. The points about weight loss and age in transgenic mice are hard to understand.

Thank you for pointing this out, we agree that this is hard to understand and has been removed from the manuscript.

Reference List

1 Gibson, R. J. *et al.* Irinotecan causes severe small intestinal damage, as well as colonic damage, in the rat with implanted breast cancer. **18**, 1095-1100 (2003).

2 de Vasconcelos Generoso, S. *et al.* Dietary supplementation with omega-3 fatty acid attenuates 5-fluorouracil induced mucositis in mice. **14**, 54 (2015).

3 Justino, P. F. *et al.* Regulatory role of Lactobacillus acidophilus on inflammation and gastric dysmotility in intestinal mucositis induced by 5-fluorouracil in mice. *Cancer Chemother Pharmacol* **75**, 559-567, doi:10.1007/s00280-014-2663-x (2015).

4 Trindade, L. M. *et al.* Oral administration of Simbioflora(R) (synbiotic) attenuates intestinal damage in a mouse model of 5-fluorouracil-induced mucositis. *Benef Microbes* **9**, 477-486, doi:10.3920/bm2017.0082 (2018).

Figures are adapted from

Hytting-Andreasen, R. et al. Endogenous glucagon-like peptide- 1 and 2 are essential for regeneration after acute intestinal injury in mice. PLoS One. 13 (6), e0198046, doi:10.1371/journal.pone.0198046, (2018).

Supplemental Figure 1