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Rodent Model of Intestinal Ischemia-Reperfusion Injury via Occlusion of the Superior Mesenteric Artery

Lucie Henein[1,2], **Randy Clevenger**[1], **Karen Keeran**[1], **Lauren Brinster**[3]

[1] Animal Surgery and Resources Core, National Heart, Lung, and Blood Institute, National Institutes of Health [2] College of Veterinary Medicine, Mississippi State University [3] Division of Veterinary Resources, Office of Research Services, National Institutes of Health

Corresponding Author

Lucie Henein
lmh675@msstate.edu

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Abstract

Intestinal ischemia-reperfusion injury (IRI) is associated with a myriad of conditions in both veterinary and human medicine. Intestinal IRI conditions, such as gastric dilatation volvulus (GDV), mesenteric torsion, and colic, are observed in animals such as dogs and horses. An initial interruption of blood flow causes tissues to become ischemic. Although necessary to salvage viable tissue, subsequent reperfusion can induce further injury. The main mechanism responsible for IRI is free radical formation upon reperfusion and reintroduction of oxygen into damaged tissue, but there are many other components involved. The resulting local and systemic effects often impart a poor prognosis.

Intestinal IRI has been the subject of extensive research over the past 50 years. An *in vivo* rodent model in which the base of the superior mesenteric artery (SMA) is temporarily ligated is currently the most common method used to study intestinal IRI. Here, we describe a model of intestinal IRI utilizing isoflurane anesthesia in 21% O_2 medical air that yields reproducible injury, as demonstrated by consistent histopathology of the small intestines. Tissue injury was also assessed in the colon, liver, and kidneys.

Introduction

Ischemia-reperfusion injury (IRI) can occur in any organ and involves two sequential components. An initial cessation of blood flow causes affected tissues to become ischemic and then subsequent reperfusion induces further cell injury. Damage from the reperfusion often exceeds that caused by ischemia[1]. The pathophysiology of IRI involves a complex cascade of events, the most notable of which is free radical formation upon the reintroduction of oxygen, which occurs during reperfusion[2]. Activation of the inflammatory cells and cytokines also plays a role[2]. In cases of intestinal IRI, bacterial translocation into the bloodstream following endothelial damage can lead to systemic inflammatory response syndrome[2]. If the damage due to IRI is severe

enough, resultant systemic effects can lead to multi-organ failure[3].

Cases of intestinal IRI are associated with high morbidity and mortality[4,5,6]. Intestinal IRI is associated with many pathologic conditions and surgical procedures in both veterinary and human medicine. In veterinary medicine, animals are especially prone to intestinal IRI conditions, such as gastric dilatation volvulus (GDV), mesenteric torsion, and colic[7,8]. In humans, IRI is a major and frequently occurring problem in abdominal aortic aneurysm surgery, strangulated hernias, acute mesenteric ischemia, volvulus, trauma, shock, neonatal necrotizing enterocolitis, and small bowel resection or transplantation[9].

Most *in vivo* rodent studies of intestinal IRI involve occlusion of the base of the superior mesenteric artery (SMA), the branch of the abdominal aorta that supplies blood to the majority of the small intestines and the proximal portion of the large intestines[10,11,12]. Despite this model's widespread use and relative simplicity, a detailed protocol using inhalant anesthesia in 21% O_2 medical air has not been published. The lack of a standard protocol poses difficulty for researchers who are unfamiliar with the procedure and prevents consistency across studies. We demonstrate the steps necessary to conduct the surgical model of intestinal IRI in 8-14-week-old male and female Swiss Webster mice. This model of intestinal IRI yields reproducible injury, as demonstrated by consistent histopathology.

Protocol

The procedures described here were approved by the National Heart, Lung, and Blood Institute Animal Care and Use Committee at the National Institutes of Health and conform to the policies outlined in The Public Health Service Policy on Humane Care and Use of Laboratory Animals, The Animal Welfare Act, and the Guide for the Care and Use of Laboratory Animals.

1. Surgical setup

1. Follow aseptic procedures. Don a mask, hair cover, and clean jumpsuit/lab coat/surgical scrubs.
2. Prepare the following sterilized materials: surgical instruments (see **Table of Materials**), warm saline, cotton swabs, gauze, surgical staples, surgical drapes, and gloves. Also obtain surgical tape, which does not need to be sterilized. Sterilize the materials with either autoclave or ethylene oxide sterilization techniques.
3. Place a heated circulating water blanket in the surgery area and cover it with a sterile towel or drape.
4. Use a precision isoflurane vaporizer, pressurized medical air (21% O_2), and a Bain non-rebreathing circuit with a nose cone designed for mice to provide surgical anesthesia.

2. Animal preparation

1. Anesthetize the mouse in an induction chamber by delivering 2%-4% isoflurane with 21% O_2 medical air at a rate of 0.5 L/min for each liter of chamber volume.
   NOTE: It is preferable to use 21% O_2 medical air over 100% O_2 for this particular model, as saturating the blood with O_2 may interfere with the IRI.
2. Remove the mouse from the chamber and move it to a clean surface separated from the surgery area. Fit it with a nose cone delivering 1.5% isoflurane with 21% O_2 medical air.

3. Inject 1 mg/kg buprenorphine subcutaneously into the dorsal cervicothoracic area.
4. Inject 200-600 IU/kg heparin intraperitoneally to prevent thrombus formation during the period of occlusion.
5. Apply ophthalmic ointment to the eyes to prevent corneal damage.
6. Remove hair from the ventral abdomen using clippers.
7. Move the mouse onto the heated water blanket in the surgery area. Again, fit it with a nose cone delivering 1.5% isoflurane with 21% O_2 medical air to achieve a surgical plane of anesthesia.
8. Position the mouse in dorsal recumbency and secure the limbs to the table with surgical tape.
9. Monitor the animal's body temperature rectally using a rodent-specific thermometer. Keep the body temperature at 36.5 ± 0.5 °C for the entire duration of the surgery.
10. Disinfect the ventral abdomen using sterile gauze soaked in either chlorhexidine scrub or povidone-iodine scrub, followed by 70% alcohol. Repeat this sequence three times, alternating between the scrub and the alcohol. A new set of scrubs and alcohol gauzes should be used each time.
    1. Apply the scrub and alcohol in a circular motion, starting with small circles at the center of the surgical site and gradually working toward the edges by increasing the size of the circles. Discard the gauze once the edge of the surgical site has been reached. Do not scrub backward from edge to center.
11. Perform a toe pinch test (pedal reflex) to ensure the animal is fully anesthetized.
12. Don sterile gloves. Aseptically drape the surgical site.

3. Surgery and ischemia

1. Make a 3-5 cm ventral midline abdominal incision in the skin using a #15 scalpel blade, dissect it free from the underlying muscle fascia, and reflect it laterally. Continue the incision through the abdominal wall along the linea alba using either micro dissecting scissors or spring-loaded micro scissors and place a retractor in position.
2. Place sterile gauze pads moistened with warm sterile saline around the operating area.
3. Remove the small intestine from the abdominal cavity, flip it cranially and to the animal's left, and place it on the moistened pads. Place another moistened gauze pad over the tissues to prevent desiccation. Periodically drip warm sterile saline on the gauze to keep the tissues moist.
4. Isolate the SMA, which is located ventral to the inferior vena cava, caudal to the celiac artery, and cranial to the renal artery.

   NOTE: **Figure 1** shows the location of the SMA where it is isolated during surgery. The SMA normally lies ventral to the inferior vena cava and extends toward the right. When the intestines are exteriorized and flipped to the left during the surgery, the SMA lies to the left of the inferior vena cava.
5. Place an atraumatic microvascular clip across the base of the SMA where it branches off the abdominal aorta, ensuring the clip does not occlude the superior mesenteric vein.
6. Verify ischemia of the small intestine by noting the color change from pink to pale-white and the loss of mesenteric pulsation.

7. Return the viscera to its original position inside the abdominal cavity for the duration of the ischemic period. Remove the retractor and cover the incision with moist gauze. Periodically add warm sterile saline to the gauze to prevent desiccation and maintain body temperature.
8. After a 45 min period of ischemia (the beginning of which is marked by the initial application of the clip), remove the occluding clip. Verify the restoration of blood flow by observing a mesenteric pulsation and flushed color.
9. Apply warm sterile saline intraperitoneally just prior to final closure to maintain appropriate hydration.
10. Close the abdominal muscles with a 6-0 polyglactin 910 suture. Administer bupivacaine (up to 2 mg/kg) along the muscle incision line for pain relief. Close the skin with surgical staples or wound clips.

4. Recovery and reperfusion

1. Return the mouse to a warm chamber or cage on a circulating water blanket, hand warmer, or other appropriate heat source. Deliver 21% O_2 at a flow rate of 0.5 L/min for each liter of chamber volume. Let the mouse recover here for 90 min. Monitor the mouse every 5-10 min for signs of pain or distress, such as hunched posture, squinting, and reluctance to move.

5. Euthanasia and blood collection

1. At the end of the 90 min recovery period, return the mouse to the induction chamber and deliver 2%-4% isoflurane with 21% O_2 at a rate of 0.5 L/min of chamber volume to re-induce full anesthesia.
2. Transfer the animal back to the surgery area and fit it with a nose cone delivering 2%-4% isoflurane with 21% O_2 to achieve deep anesthesia.

   NOTE: CO_2 is not an appropriate method of euthanasia for this procedure, as it induces physiologic changes which may interfere with ischemic injury or tissue analytes[13].
3. Re-open the ventral midline incision and perform a terminal bleed by collecting as much blood as possible from the abdominal vena cava using a 23 G needle and syringe. Expect to collect between 0.3-0.5 mL of blood (less in mice who have undergone IRI, more in those who received sham laparotomy).

   NOTE: The purpose of the terminal bleed is to aid in humane euthanasia and to collect and preserve blood for future testing (i.e., serum chemistry, PCR, ELISA).
4. Following blood collection, the abdominal aorta is severed to allow for complete exsanguination.
5. Perform either cervical dislocation or thoracotomy as a secondary measure to ensure successful euthanasia.

6. Tissue processing for histology

1. After euthanasia, collect the desired tissues. Ensure that the tissue processing is done promptly, as autolysis begins immediately after death[14,15].
   1. Intestines: Collect the entire length of the small intestine and large intestine. Discard the cecum.
   2. Liver: Collect the left lateral, left median, and right median lobes.
   3. Kidneys: Collect both kidneys. By convention, the left kidney is cut longitudinally, and the right is cut as a cross section at the time of necropsy.

      NOTE: The colon, liver, and kidneys may be used for assessing multi-organ failure or other systemic effects of IRI. The small intestines are used to

assess the primary injury. It is not necessary to keep track of individual sections of the liver lobe and kidneys, as each organ will be analyzed and scored as one unit. The intestinal segments, however, should be kept separate and then labeled and scored individually.

2. Divide the intestine into four sections: duodenum, jejunum, ileum, and colon. Ensure that the three small intestinal segments are equal in length. Do this by folding the small intestines into a "Z" shape, where the top line is the duodenum, the middle line is the jejunum, and the bottom line is the ileum. It is important to keep track of the proximal versus the distal end.
3. Flush the lumen of the intestinal segments with saline using a 10 mL syringe affixed with a 20 G angio-catheter.
4. Prior to making sections, lay each intestinal segment flat with the luminal side facing up. Using a 3 mL syringe affixed with a 27 G needle and generously apply 10% buffered formalin dropwise to coat the entire length of the mucosa. Then, roll each intestinal segment individually and place in separate, labeled tissue cassettes.
    1. To roll, lay each segment flat with the luminal side facing up, then roll circumferentially around a toothpick. The proximal portion should form the inner part of the roll. The lumen should face the inside/ center. Try to roll as gently as possible to avoid compressing the villi.
    2. When rolled, the intestine should look like a Swiss roll. Place the Swiss roll spiral face-up inside the cassette.
5. Place the tissues in labeled vials filled with 10% buffered formalin to fix them at room temperature. Over-fixing is better than under-fixing. The vials should be large with plenty of formalin-at least 20x more fixative than tissue.
    1. Intestines: Place the four cassettes together in a specimen cup. Fix for 24-48 h.
    2. Liver: Place the liver lobes together in a 50 mL conical tube. Fix for 24-48 h.
    3. Kidneys: Place the kidneys together in a 50 mL conical tube. Fix for 48-72 h.

       NOTE: Untrimmed kidneys take longer to fix than trimmed kidneys. To shorten the fixation time to 24-48 h, the kidneys may be cut along the median plane, longitudinally (left kidney) and transversely (right kidney), and placed in cassettes before being deposited in the formalin.
6. After the tissues have been fixed in formalin for the designated time, rinse with phosphate-buffered saline (PBS) or distilled water and transfer to labeled vials filled with 70% EtOH. The tissue can be stored in the EtOH indefinitely at room temperature while awaiting histology.
    1. Intestines: Place the four cassettes together in a specimen cup.
    2. Liver: Place the liver lobes together in a 50 mL conical tube.
    3. Kidneys: Place the kidneys together in a 50 mL conical tube.
7. When ready, have the tissues processed onto glass slides using hematoxylin and eosin (H&E) staining. Trim the formalin-fixed tissues and then embed them in paraffin. Mount five-micron sections on the slides and stain with H&E.

7. Tissue scoring

1. Tissue scoring should preferably be performed by experienced personnel who are blinded to the sample groups.
2. Intestinal ischemia is scored using the Chiu/Park scoring system[17].
3. Kidney damage is scored using the Jablonski scoring system[18,19].
4. Liver damage is scored using the Suzuki scoring system[20,21].

   NOTE: There are many scoring systems currently in use for assessing tissue damage in rodent models of intestinal IRI. The scoring systems used in this study were selected to minimize arbitrary estimation and to maximize intentional qualitative assessment (**Table 1**).

Representative Results

We demonstrated a model of intestinal IRI in mice that yielded consistent and reproducible results. The small intestine, proximal colon, kidneys, and liver were sectioned and stained with H&E. A veterinary pathologist graded tissue injury using the scoring systems previously mentioned (**Table 1**). Statistical analysis was performed using single factor analysis of variance (ANOVA) followed by Tukey's post hoc with pairwise comparisons, which determined whether or not there was a significant difference in the data within and across groups. A *p*-value less than or equal to 0.05 was considered the cutoff for establishing statistical significance. All statistical tests and graphing were carried out in a spreadsheet software (e.g., Microsoft Excel) with the Real Statistics Resource Pack add-on. Data are presented as the mean ± standard error of the mean (SEM).

Microscopic lesion scores of the three small intestinal segments (duodenum, jejunum, and ileum) were significantly increased for animals undergoing intestinal ischemia-reperfusion injury (IRI; N = 7) versus those that underwent sham laparotomy (Sham; N = 6) (**Figure 2** and **Figure 3**). The standard error for these data was narrow, demonstrating consistency of the results within and across groups. Each intestinal segment in the Sham group yielded the exact same average Park/Chiu score of 0.83. The SEM for the duodenum, jejunum, and ileum in the Sham group was 0.31, 0.40, and 0.31, respectively. The average Park/Chiu scores for the duodenum, jejunum, and ileum in the IRI group were 4.07 ± 0.44, 4.14 ± 0.46, and 5.14 ± 0.40, respectively.

In this study, 50% (3/6) of the initial mice that underwent 60 min ischemia and 120 min reperfusion (60/120 group) died. Two of the three mice were submitted for necropsy. Both mice had epithelial necrosis, congestion, and hemorrhage of the small intestine. In addition, the mice had lymphocytolysis, a nonspecific change associated with physiologic stress. Neither mouse had lesions in the heart, lung, liver, or kidneys. Shortening the times to 45 min ischemia and 90 min reperfusion and adding in 400 IU/kg heparin (45/90/H group) lowered the mortality to 20% (1/5) without changing the intestinal injury scores (**Figure 4**). The mean Park/Chiu score for the 60/120 group was 4.56 ± 0.38 (N = 3), and the mean score for the 45/90/H group was 4.375 ± 0.38 (N = 4).

Microscopic findings indicative of injury in the proximal colon, liver, and kidney were not seen in either the 60/120 mice or the 45/90/H mice.

Table 1: Scoring systems for the intestines, kidneys, and liver. Intestinal damage was graded using the Chiu/Park system[17]. Kidney damage was graded using the Jablonski scoring system[18,19]. Liver damage was graded using the

Suzuki scoring system[20,21]. This table is adapted with permissions from scoring systems presented in Quaedackers et al.[17], Du et al.[19], and Behrends et al.[21]. Please click here to download this Table.

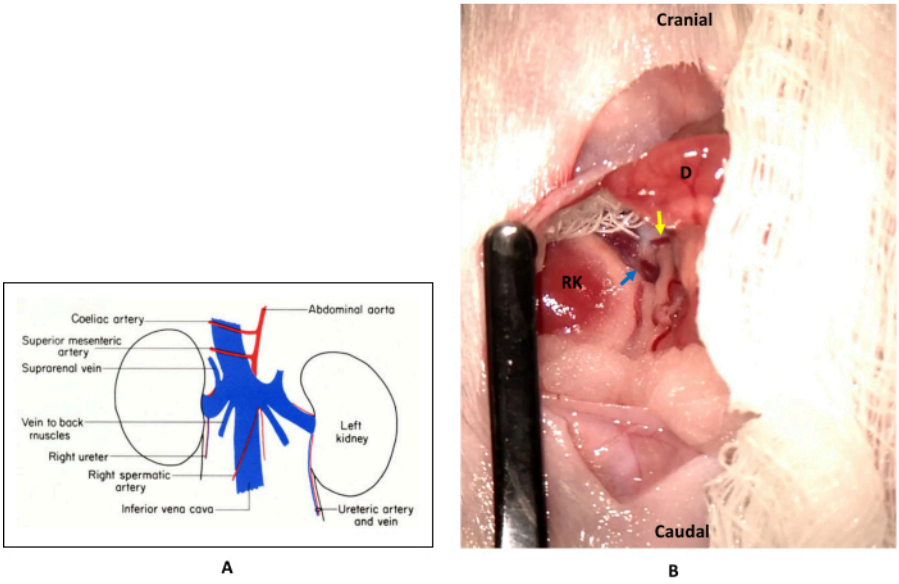

Figure 1: Location and isolation of the superior mesenteric artery (SMA). (**A**) Normally, the SMA lies ventral to the inferior vena cava and extends toward the animal's right. It is situated between the celiac artery and the renal artery. This figure is adapted with permission from The Anatomy of the Laboratory Mouse by Margaret Cook (1965)[22]. (**B**) In this procedure, the intestines are exteriorized and flipped to the left (covered with moistened gauze in this picture), so the SMA (yellow arrow) lies to the left of the inferior vena cava (blue arrow). Abbreviations: RK = right kidney; D = duodenum. Please click here to view a larger version of this figure.

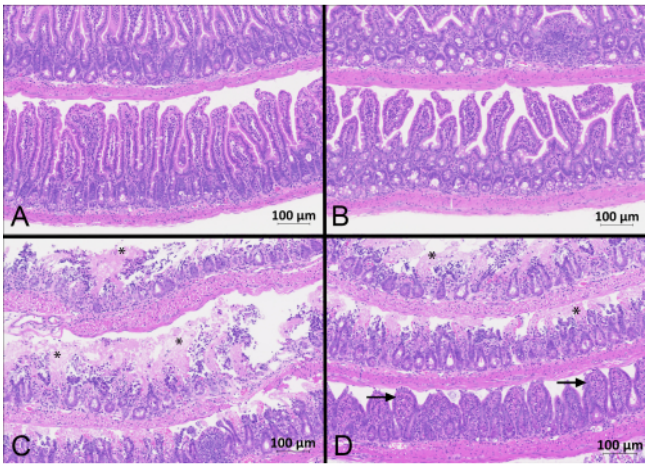

Figure 2: Small intestinal segments stained with hematoxylin and eosin. Sections of jejunum (**A**) and ileum (**B**) from mice in the Sham group featured villi that were long and thin without distortion. Sections of jejunum (**C**) and ileum (**D**) from mice in the IRI group featured areas of necrosis (asterisks) and hemorrhage with blunting and distortion of the remaining villi (arrows). The photos are from mice that underwent 45 min ischemia and 90 min reperfusion and received 400 IU/kg heparin. The photos were taken at 20x magnification with 10% zoom. Scale bar = 100 µm. Please click here to view a larger version of this figure.

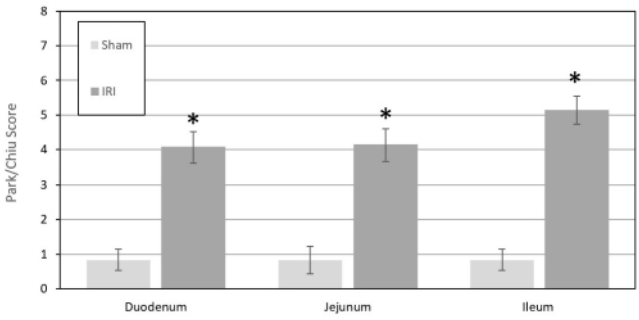

Figure 3: Park/Chiu scores for small intestinal segments. Microscopic damage to all three intestinal segments (duodenum, jejunum, and ileum) for animals undergoing intestinal ischemia-reperfusion injury (IRI) was significantly increased compared to those that underwent sham laparotomy (Sham). * $p < 0.05$ for IRI versus Sham. Please click here to view a larger version of this figure.

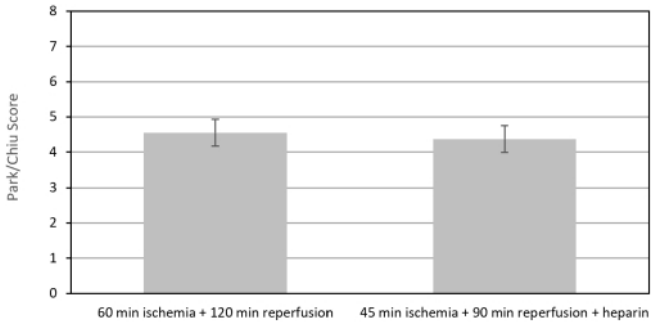

Figure 4: Park/Chiu scores for small intestines undergoing 60 min ischemia and 120 reperfusion versus 45 min ischemia and 90 min reperfusion with 400 IU/kg heparin. Decreasing the times from 60 min ischemia and 120 min reperfusion (60/120) to 45 min ischemia and 90 min reperfusion with 400 IU/kg heparin (45/90/H) did not create a statistically significant difference in Park/Chiu injury scores of the small intestines of mice in the IRI group. It did, however, reduce mortality from 50% to 20%. Please click here to view a larger version of this figure.

Discussion

Despite the widespread use of this intestinal IRI model, it is not without its limitations. For instance, sole occlusion of just the base of the SMA does not completely obstruct blood flow to the intestine. This is likely due to extensive collateral circulation in the mesentery, which can draw blood from neighboring branches of the abdominal aorta. In one study in cats, SMA occlusion decreased blood flow by 35% in the proximal duodenum, 61% in the distal duodenum, 71% in the jejunum and ileum, and 63% in the proximal colon. Blood flow was not reduced in the mid and distal colon, which receive much of their circulation from the inferior mesenteric artery[23]. In rodents, the jejunum and ileum are most often cited as the intestinal segments which incur the most significant tissue injury following SMA occlusion[9].

A wide range of ischemia times after SMA occlusion have been cited in the literature, from 1 to 90 min or more. Different ischemic times result in different levels of reperfusion injury; Park et al. observed reperfusion injury when the ischemic interval was between 40 and 60 min, but not when the ischemic interval was shorter or longer[24]. Such results suggest that shorter times do not produce enough ischemia to incite reperfusion injury, while longer times damage the tissue so severely that it is impossible to demonstrate the reperfusion injury that follows. In addition, longer ischemic times carry the risk of increased mortality. As seen in our study, 50% (3/6) of the initial mice that underwent 60 min ischemia died after only 90 min of reperfusion. Shortening the ischemia time to 45 min lowered the mortality to 20% (1/5) without changing the tissue injury scores. Based on our study, it appears that the ideal window of ischemic damage can be attained by SMA occlusion for about 45 min.

Another variable is the reperfusion time before tissue collection. As with ischemia times, reperfusion times vary widely across studies, from 60 min to over 24 h. Several papers have reported that the intestinal mucosa incurs maximal morphologic damage at 2 to 3 h of reperfusion, with

complete repair achieved at 24 h[25,26,27]. Collecting tissue before this 2 to 3 h window risks not capturing the full extent of the reperfusion injury, while tissues harvested closer to 24 h will have already started the repair process. We initially opted for a reperfusion time of 120 min, but then changed to 90 min in an effort to lower mortality. This change did not change tissue injury results, suggesting that a 30 min deviation from the 2 to 3 h window is acceptable.

Oxygen concentration is also an important variable in the development of IRI. Wilding et al. found that, compared to mice receiving 21% O_2, those anesthetized with isoflurane delivered with 100% O_2 experienced ventilation-perfusion mismatch due to atelectasis. In the same study, rats receiving 100% O_2 developed acute respiratory acidosis and elevated mean arterial pressure[28]. Such physiologic changes are best avoided when inducing an injury such as IRI, in which a number of systemic factors are involved. Thus, 21% O_2 seems to be more appropriate than 100% O_2 as the carrier gas for isoflurane delivery.

The use of heparin in this protocol is open to debate. Heparin is known to have anti-coagulative and anti-inflammatory effects[29]. We found that changing from 60 min ischemia and 120 min reperfusion to 45 min ischemia and 90 min reperfusion with 400 IU/kg heparin did not change microscopic intestinal injury but did lower mortality. One possible explanation is that heparin prevented fatal thromboembolism to distant organs such as the lungs and brain, however we did not find evidence of this on necropsy by gross or microscopic examination of the initial two mice that died. Using shorter ischemia and reperfusion times without heparin may be just as effective at reducing mortality. If that were the case, it would be prudent to forego the use of heparin to minimize interference with IRI. However, including heparin in the protocol may be appropriate for those wishing to model surgical causes of IRI, as surgical patients often receive heparin peri-operatively.

Isoflurane has been shown to have tissue protective effects in cases of intestinal inflammation and ischemia, and its use may interfere with a clinically relevant IRI model[30,31,32]. However, organofluorine inhalants (i.e., isoflurane, sevoflurane) are commonly used anesthetics in both veterinary and human medicine. In addition, the length of anesthesia required for this protocol exceeds 120 min, and thus an inhalant is more appropriate than a shorter-acting injectable which would need to be re-dosed.

No microscopic lesions were present in the proximal colon, liver, or kidney. The lack of microscopic changes was perhaps due to the relatively short 90 to 120 min reperfusion time. In addition, the proximal colon has a blood supply from the inferior mesenteric artery. However, a lack of visible damage does not rule out systemic injury. Reverse transcription-quantitative polymerase chain reaction (RT-qPCR) is likely a better methodology to demonstrate systemic injury by measuring inflammatory cytokines such as TNF-α.

Several variations of this intestinal IRI model have been developed over the years. In 1990, Megison et al. demonstrated that occluding collateral vessels in addition to the SMA produced a more consistent reduction of mesenteric blood flow but an increase in the mortality rate[33]. A more recent study showed that, in lieu of occluding the SMA at its base, ligating its peripheral and collateral branches to induce ischemia in the distal ileum yielded reproducible injury without mortality[34]. Occlusion of the local arterial branches ensures maximal ischemia and may address the issue of multifocal, segmental reductions of blood flow seen with ligating the SMA just at its base. While this alternative method

of modeling intestinal IRI has application for research into the local tissue effects of intestinal IRI, it is unknown whether it can accurately model the systemic inflammation and multi-organ failure which can be associated with intestinal injury.

SMA occlusion is not an appropriate model for all types of intestinal IRI. Non-occlusive mesenteric ischemia, for instance, is characterized by splanchnic hypoperfusion stemming from decreased cardiac output. Therefore, this technique would not be optimal to study intestinal IRI caused by myocardial infarction, congestive heart failure, aortic insufficiency, or renal or hepatic disease[35]. Kozar et al. reported that SMA occlusion is, however, a clinically relevant model for gut IRI induced by shock[36]. Although less economical, the use of other species such as pigs may have benefits over rodents for modeling certain intestinal injury conditions. A comprehensive review by Gonzalez et al. in 2014 describes animal models currently in use for investigating intestinal IRI[9].

Despite its limitations, the technique of occluding the SMA at its base remains one of the most commonly utilized rodent models of intestinal ischemia[9]. As it only requires one vascular clamp and a basic setup, the surgery itself is quite simple. It also yields reproducible injury, as evidenced by the data presented here. SMA occlusion in rodents can reliably model occlusive causes of intestinal IRI and can have practical application in both veterinary and human medicine. As such, it is important that the procedures we have outlined here be carried out with consistency.

Disclosures

The authors of this paper have no conflicts of interest to disclose.

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