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A Murine Model of Metastatic Liver Tumors in the setting of Ischemia Reperfusion Injury

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March 27, 2019

Dear Editorial Board,

We appreciate your interest in publishing the manuscript entitled *"A Murine Model of Metastatic Liver Tumors in the setting of Ischemia Reperfusion Injury"* to the *Journal of Visualized Experiments* (JoVE59748). We are grateful for the opportunity to resubmit a revision. The manuscript has been revised to address the supportive and constructive comments from the editor. Specific responses have been addressed point by point, changes are tracked within the manuscript and their details are described below.

The manuscript has been approved by all of the authors. The revisions are being submitted electronically as instructed. We sincerely hope that the manuscript will be accepted for publishing in its present form. The authors again wish to thank the editorial office and editor for consideration of this manuscript for publication in *Journal of Visualized Experiments*.

Sincerely,

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Fellow, Complex General Surgical Oncology
Department of Surgery
University of Pittsburgh

TITLE:

Murine Model of Metastatic Liver Tumors in the Setting of Ischemia Reperfusion Injury

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KEYWORDS:

liver metastases, liver ischemia-reperfusion, laparotomy, splenectomy, murine colorectal cancer, hepatic injury, mouse model

SUMMARY:

We describe in detail a clinically relevant colorectal cancer liver metastases (CRLM) tumor model and the influence of liver ischemia reperfusion (I/R) in tumor growth and metastasis. This model can help to better understand the mechanisms underlying surgery-induced promotion of liver metastatic growth.

ABSTRACT:

Liver ischemia and reperfusion (I/R) injury, a common clinical challenge, remains an inevitable pathophysiological process that has been shown to induce multiple tissue and organ damage. Despite recent advances and therapeutic approaches, the overall morbidity has remained unsatisfactory especially in patients with underlying parenchymal abnormalities. In the context of aggressive cancer growth and metastasis, surgical I/R is suspected to be the promoter regulating tumor recurrence. This article aims to describe a clinically relevant murine model of liver I/R and colorectal liver metastasis. In doing so, we aim to assist other investigators in establishing and perfecting this model for their routine research practice to better understand the effects of liver I/R on promoting liver metastases.

INTRODUCTION:

The liver is one of the most common sites for the development of metastatic disease[1]. Mortality is almost invariably attributable to complications associated with tumor growth in the liver. In patients with metastatic solid tumors in the liver, surgery remains a crucial intervention for disease control and a possible curative approach. However, the vast majority of patients ultimately present with recurrent disease, predominantly in the liver[2,3]. During hepatic surgery, intraoperative bleeding is common, often necessitating blood transfusion and different technical approaches for control of bleeding, including vascular clamping methods. However, such measures cause hepatic ischemia/reperfusion (I/R) to the liver tissue. The adverse effects of I/R

on hepatocellular function have been well documented. The liver I/R insult ignites inflammatory cascades during the restoration of blood flow via inflammatory pathways[4]. Not only does liver I/R injury contribute to liver failure, but current evidence also shows that I/R injury stimulates tumor cell adhesion, and promotes the incidence of metastases formation and the growth of existing micrometastatic disease[5]. We have previously reported that surgical stress induces activation of immune cells which not only helps in the growth of the primary tumor, but also facilitates metastases by capturing cancer cells within the circulation[6].

Here we describe in detail a technique to establish a liver metastasis mouse tumor model. In this model, we also present a method to induce hepatic ischemia reperfusion injury which acts as a surrogate to the surgical stress present clinically during hepatectomies. The combined methods of cancer injection and hepatic I/R can successfully interpret the development of CRLM in patients who have undergone primary tumor resection.

PROTOCOL:

All animal protocols are approved by the Institutional Animal Care and Use Committee and adhered to the National Institutes of Health (NIH) Guidelines. Instruments used for any surgical procedure were thoroughly sterilized.

1. Initial preparation

1.1. Before injecting cancer cells into the mouse spleen, autoclave and sterilize all instruments to be used during the procedure.

1.2. Sterilize and/or autoclave a heating pad, surgical gloves, gauze, pairs of scissors, small clamps, vessel dilator, surgical forceps, and a needle holder.

1.3. Prepare post-operative analgesic (0.1 mg/kg of buprenorphine) to be administered after splenectomy and every 12 h for 2 days.

2. Cell culture

2.1. Ensure that cancer cells are free from mycoplasma contamination by using a mycoplasma ELISA kit.

2.2. Prepare a 500 mL solution of Dulbecco's Modified Eagle medium (DMEM) culturing medium at 4 °C for the culture of murine colorectal cancer cells (MC38). The culturing media should be supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/mL of penicillin, 100 µg/mL of streptomycin, 15 mM of HEPES and 200 mM of L-glutamine.

2.3. Culture cancer cells in a DNAse- and RNAse-free flask (75 cm^2). Incubate the cell culture in a cell/tissue humidified incubator containing 5% CO_2. Maintain temperature at 37 °C.

2.4. Once the proliferating cells reach 90–100% confluency, aspirate the old media, wash cells

with 1x phosphate-buffered saline (PBS) and then treat them with 1x trypsin (0.25%) to detach cells from the flask.

2.5. Collect cells in a 15 mL conical tube and centrifuge for 5 min at 700 x *g*.

2.6. Aspirate the media and wash with 1x PBS twice by repeated centrifugation.

2.7. Proceed to confirm cell viability by staining cells with trypan blue stain (0.4%).

2.8. Resuspend cells to a concentration of 1 x 10^6 cells/ 100 µL in 1x PBS. Pipet cells thoroughly to avoid any clumps. Keep cancer cells on ice prior to injection.

3. Injecting tumor cells

3.1. Anesthetize 8–12-week-old male (C57BL6) mice by administering ketamine (150 mg/kg) and xylazine (12 mg/kg) intraperitoneally using a 1 mL 25 G (0.5 mm x 16 mm) needle.

3.2. Shave the abdominal skin of the mice using clippers to avoid any postoperative infections.

3.3. Place mice on the magnetic fixator retraction system. Confirm that the mice are completely under the effect of anesthesia by pinching a toe or the tail.

3.4. Add saline drops into the eyes to avoid dryness during the procedure.

3.5. Scrub povidone-iodine solution (7.5%) to the shaved abdominal wall to disinfect the skin before making a surgical incision.

3.6. Initially lift the skin with the toothed forceps and make a midline incision with the help of surgical scissors. Then, lift the abdominal muscle and peritoneum to create a midline incision of approximately 3 cm length (midabdominal to xiphoid process to expose the abdominal contents. Take caution not to extend the incision beyond the xiphoid process to avoid extensive bleeding.

3.7. Place the hemostat at both sides of the incision and under the xiphoid process. Extend the abdomen by pulling the tail downward and taping it. Use the 6-inch sterile cotton tip applicator to separate and expose the spleen from the pancreatic fat tissue.

3.8. Before injection into the spleen, vortex the cancer cells to avoid any cell clumps.

3.9. Use a 0.5 mL 28 G (0.36 mm x 13 mm) insulin syringe for injection. Avoid air bubbles.

3.10. Slowly and carefully inject 100 µL of cells into the tip of the spleen. Place a cotton tip and add gentle pressure to avoid backflow into the abdominal region. A successful injection can be observed by identifying the change in the color of the liver during the injection.

3.11. Moisten a sterile gauze with 1x PBS and place it over the dissected area.

3.12. Transfer the mice onto a heating pad for 15 mins to allow cancer cells to circulate within the system.

3.13. To perform surgical ischemia and reperfusion injury, follow the steps 4.3–4.7.

3.14. To perform a splenectomy, use a hand-held cautery device. Carefully lift the spleen with smooth forceps and cauterize the splenic blood vessels to avoid excessive bleeding. Remove spleen by transecting the vessels at the cauterized section.

3.15. Immediately following the procedure, close the incision in a double layer pattern by first suturing the muscle layer and then the skin. Use 4-0 polypropylene sutures for both the abdominal wall and the skin.

3.16. Before repeating the procedure on another animal, disinfect all instruments by either spraying them with 70% isopropanol or inserting them into a bead bath.

3.17. Place mice back into original cages and look for signs of distress and post-surgical pain.

3.18. Inject post-operative analgesic (Buprenorphine 0.1 mg/kg) every 12 h for 2 days to avoid post-surgical pain.

4. Ischemia reperfusion Injury

4.1. At 5 days after the first laparotomy, anesthetize mice by administering ketamine (150 mg/kg) and xylazine (12 mg/kg) intraperitoneally using a 1 mL 25 G (0.5 mm x 16 mm) needle. Follow steps 3.3–3.4.

4.2. Scrub povidone-iodine solution (7.5%) on the shaved abdomen of the mouse to disinfect the skin and perform a midline laparotomy as described above in step 3.6.

4.3. Using two moistened cotton tips, gently move the intestine from the cavity to expose the associated structures, including the portal vein. Dissect the liver hilum free of the surrounding tissue.

4.4. Lift up the median and left lateral lobes against the diaphragm. Separate the quadrate lobe from the left lateral lobe by dissecting the liver hilum with the spring scissors using an operating microscope to allow clear visibility towards the portal triad structure.

4.5. Place a small moist cotton swab between the median lobe and right lateral lobe to create sufficient space for clamping. Using the vessel dilator forceps, carefully pass the 10 cm thread (4.0 polypropylene suture) to lift the portal triad. Occlude all structures in the portal triad (hepatic artery, portal vein, and bile duct) to the left and median liver lobes by placing a microvascular

clamp using a micro-serrefine clamp applicator with lock.

4.6. If the lobes do not show significant blanching, readjust the clamp by removing and reapplying.

NOTE: If the immediate blanching of the liver does not occur even after readjusting the clamp, carefully consider whether or not to proceed with the I/R.

4.7. Remove the small cotton swab placed between the median and right lateral lobes. Gently replace the intestine into the abdominal cavity. Cover the abdominal wall with a moist gauze (soaked with 1x PBS) and cover with a plastic wrap to minimize evaporative loss.

4.8. Place the mouse on the heating pad and apply the clamp for a period of 60 min.

4.9. Throughout the ischemic interval, seek evidence of ischemia injury by visualizing the pale blanching of the right medial and left medial and lateral lobes.

4.10. Initiate reperfusion by removing the clamps after the 60 min period.

NOTE: Evidence of reperfusion can be observed by an immediate color change of the median and left lateral lobes.

4.11. Immediately following reperfusion, close the incision with a double layer suture pattern by first suturing the muscle layer and then the skin. Use the 4-0 polypropylene suture with the help of a needle holder to close the abdominal wall and the skin.

4.12. Before repeating the procedure on another animal, disinfect all instruments by either spraying them with 70% isopropanol or inserting them into a heated bead bath.

4.13. Place mice back into original cages and look for signs of distress and post-surgical pain.

4.14. Inject post-operative analgesic (0.1 mg/kg of buprenorphine) every 12 h for 2 days to avoid post-surgical pain.

4.15. For liver I/R sham mice, perform laparotomy, hilum dissection and abdominal sutures.

NOTE: The role of surgical stress influencing the establishment of liver metastases can be investigated through two different experimental designs. The above protocol (**Model-1**) is used to establish micrometastatic liver disease and study the effect of liver I/R on their growth (**Figure 1A**)**.** Alternatively, liver I/R and tumor injection can be performed concurrently (**Model-2**) to study the effect of I/R injury in the establishment of new metastatic foci (**Figure 1B**). To do this, inject cancer cells into the spleen as described above and allow them to circulate for 15 min. Perform liver I/R or sham surgery after the circulating period for 60 min. Perform lateral splenectomy 60 min later, and then close the laparotomy incision.

5. Assessment of operated mice

5.1. Allow mice 30–60 min of recovery time from anesthesia. Constantly monitor mice and do not leave them unattended until complete recovery.

5.2. Look for distress signs such as hunched back, closed eyes, slow movement, and failure to groom. Treat accordingly until mice return to their normal activity.

6. Assessment of liver ischemia reperfusion injury

6.1. Immediately after applying the clamp, make sure the pale blanching of the median and left lateral lobes occurs compared to the caudate and quadrate lobes.

6.2. Assess liver ischemia injury by measuring serum alanine transaminase (sALT), serum aspartate transaminase (sAST) and serum lactate dehydrogenase (sLDH) levels. The blood can be drawn from the facial vein to extract serum 3–6 h after the initiation of reperfusion. Perform liver histology to analyze the percent tumor area within the ischemic lobe.

REPRESENTATIVE RESULTS:

All wildtype (C57BL6) mice (n = 20) were subjected to the liver metastases model using the protocol described above. All injected mice with or without ischemia reperfusion injury survived until the date of sacrifice. The schematic diagram **Figure 1A** of a cancer-injected liver illustrates the clamping of the portal triad (hepatic artery, portal vein, and bile duct) which induces a partial liver ischemic (70%) insult towards the median and left lateral lobes. An increase in the number of liver metastases can be observed within 2–3 weeks post ischemia reperfusion injury. Mice injected with MC38 cancer cells were randomly divided into sham and I/R groups. As shown in **Figure 1B**, the first group of mice underwent splenectomy 15 min after the cancer injection. Liver ischemia reperfusion surgery was performed 5 days after the injection. This model allows the circulating cancer cells (CCs) to establish within the organs. **Figure 2A** shows that surgical stress significantly increased the amount of pre-established micrometastases within the liver. The second group (**Figure 1C**) underwent surgical I/R 15 min after the cancer injection. The reperfusion was induced by removing the microvascular clamp 60 min after the application. The concurrent influence of the surgical stress leads (**Figure 2B**) to the capture of recently injected cancer cells within the liver establishing a micrometastatic foci. This significantly increased the number of metastatic nodules in the liver.

FIGURE LEGENDS:

Figure 1: A schematic representation of the experimental design. (**A**) The schematic diagram of a cancer injected liver illustrates the clamping of the portal triad (hepatic artery, portal vein, and bile duct) which induces a partial (70%) liver ischemic insult towards the median and left lateral lobes. An increase in the number of liver metastases can be observed in the ischemic lobes within 2–3 weeks after the reperfusion. Initially, mice were subjected to intrasplenic injection of MC38

colorectal cancer in both tumor capture (**B**) and tumor growth (**C**) model. Sham mice were also subjected to laparotomy without the application of microvascular clamps. Two–three weeks after the I/R, mice were sacrificed, and liver tissue was harvested.

Figure 2: Representative images of mice injected with murine cancer. (**A**) Representative liver metastasis image of a tumor growth model (Model-1) showing a significant increase in the gross tumor nodules at the surface of the liver after inducing hepatic I/R compared to non-I/R group. (**B**) Similarly, in the setting of tumor capture model (Model-2), liver I/R showed a significant increase in tumor nodules 2 weeks after I/R compared to non-I/R group. *, $P < 0.05$. Results are expressed as the mean ± standard deviation. Group comparisons were performed using Student's t test (n = 5/group).

DISCUSSION:

The animal model described in this manuscript is based upon two major approaches. The first is to recognize the ability of cancer cells to localize and proliferate in the liver lobes. The second is to study the effect of hepatic ischemia reperfusion injury influencing the tumor growth and metastases. This model permits the relevant study of liver metastases in the absence of secondary metastases in an immunocompetent mouse. The model is useful in addressing the questions of metastatic efficiency, such as cell survival extravasations and proliferation.

In the first model, cancer cells are injected first and micrometastatic disease is allowed to form. Subsequently, liver I/R is performed 5 days later. This model is important when studying the effect of surgery on already established micrometastatic disease. Although imaging has significantly improved in the past decade, there is still the possibility of the presence of micrometastatic disease that may not be detected by imaging and is left behind after a planned liver resection with the intent of cure. This residual microscopic disease is affected by the inflammatory changes accompanying surgery, specifically liver I/R, and the growth is exponentially increased. On the other hand, in the second model liver I/R and tumor injection are performed at the same time. This model focuses on the effects of liver I/R on the circulating cancer cells and the establishment of new metastatic foci. During liver surgery, the manipulation of the tumor releases tumor cells into circulation. Although most of the circulating cells are taken care of by the host's immune surveillance, a number of cells can establish metastatic foci. This second model is designed to study this phenomenon.

Animal models, such as orthotopic liver injection[7] and tail vein injection[8], may not be anatomically feasible for such studies. It has been shown that the tail vein injection usually results in an increased metastasis of the lung compared to the liver. The orthotopically injected cancer model has an increased risk of liver injury influencing the microenvironment for the tumor to grow. As an alternative to splenic injection of tumors, the portal vein can also be utilized. The portal vein injection has been a well-established metastatic model in studying liver metastases[9–11]. The injection of cancer cells through the portal vein does not compromise the removal of the spleen compared to the model described above. This indeed will avoid the immune consequences. However, portal vein injection has an increased risk of excessive bleeding due venous tearing (at the site of injection) and thrombosis during or after the application of the

microvascular clamp at the portal triad. These risks are exponentially increased when both tumor injection and clamping are done on the same day. Our group has performed both methods and we have obtained similar results[9, 12]. We do acknowledge that the portal vein injection demands higher technical skills when done at the same time as clamping and is associated with higher complications. Both methods are valid to study liver metastases.

There are numerous important aspects that need to be considered before and during the entire procedure. The use of cancer cells specifically with the same species background is recommenced prior to injection. The cell number is also important to consider in this study, as small number of cells may not be sufficient to complete the study in a short period of time (3 weeks). Increasing the number of cancer cells should be avoided since it may cause an embolism effect leading to thrombosis and death of the rodent. The model described in this manuscript with cell concentration of 1×10^6 is specific to the MC38 cell line and has allowed us to observe a significant difference in the tumor growth stimulated by the effect of surgical ischemia reperfusion injury. We highly recommend trying different concentrations of cancer cells depending on the specific experiment with the desired cancer cell line of interest. Similarly, labeling of cancer cells could be very useful in many metastasis studies. This would provide an idea regarding the percentage of cells that are able to seed and proliferate. Furthermore, proper application of the portal clamp to induce hepatic ischemia injury is very important in this model. Inability to completely block the blood flow may lead to less or no impact on the cancer cells. As described in the methods, it is important to make sure that the blanching of the liver lobes occurs after applying the microvascular clamps. Finally, coagulating vessels properly via cautery is crucial to avoid internal bleeding. In our experience, especially with the use of electrocautery, bleeding from the splenic bed is extremely uncommon and since we perform a repeat laparotomy only 5 days after the first operation, the amount of adhesions is minimal. However, if bleeding is encountered, this may pose a more difficult second operation. If bleeding does occur after the splenectomy, this may indicate that circulating cancer cells may also have implanted in the peritoneal cavity and thus may affect the results of the experiments. It is advised to use caution when handling this issue, because it may affect the experiments and results. Careful thought must be given to determine whether or not to proceed with I/R in these mice.

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DISCLOSURES:
The authors disclose no conflicts of interest that pertain to this work.

REFERENCES:
1. Riihimäki, M., Hemminki, A., Sundquist, J., Hemminki, K. Patterns of metastasis in colon and rectal cancer. *Scientific Reports*. **6** (1), 29765, doi: 10.1038/srep29765 (2016).
2. Oki, E. et al. Recent advances in treatment for colorectal liver metastasis. *Annals of gastroenterological surgery*. **2** (3), 167–175, doi: 10.1002/ags3.12071 (2018).
3. Wolpin, B.M., Mayer, R.J. Systemic treatment of colorectal cancer. *Gastroenterology*. **134** (5), 1296–1310, doi: 10.1053/j.gastro.2008.02.098 (2008).

4. van Golen, R.F., Reiniers, M.J., Olthof, P.B., van Gulik, T.M., Heger, M. Sterile inflammation in hepatic ischemia/reperfusion injury: present concepts and potential therapeutics. *Journal of Gastroenterology and Hepatology*. **28** (3), 394–400, doi: 10.1111/jgh.12072 (2013).
5. Demicheli, R., Retsky, M.W., Hrushesky, W.J.M., Baum, M., Gukas, I.D. The effects of surgery on tumor growth: a century of investigations. *Annals of Oncology*. **19** (11), 1821–1828, doi: 10.1093/annonc/mdn386 (2008).
6. Tohme, S. et al. Neutrophil Extracellular Traps Promote the Development and Progression of Liver Metastases after Surgical Stress. *Cancer Research*. **76** (6), 1367–1380, doi: 10.1158/0008-5472.CAN-15-1591 (2016).
7. Tseng, W., Leong, X., Engleman, E. Orthotopic mouse model of colorectal cancer. *Journal of Visualized Experiments*. (10), 484, doi: 10.3791/484 (2007).
8. Elkin, M., Vlodavsky, I. Tail vein assay of cancer metastasis. *Current Protocols in Cell Biology*. **19** (19.2), doi: 10.1002/0471143030.cb1902s12 (2001).
9. Thalheimer, A. et al. The intraportal injection model: A practical animal model for hepatic metastases and tumor cell dissemination in human colon cancer. *BMC Cancer*. **9** (1), 29, doi: 10.1186/1471-2407-9-29 (2009).
10. Frampas, E., Maurel, C., Thedrez, P., Remaud-Le Saëc, P., Faivre-Chauvet, A., Barbet, J. The intraportal injection model for liver metastasis: Advantages of associated bioluminescence to assess tumor growth and influences on tumor uptake of radiolabeled anti-carcinoembryonic antigen antibody. *Nuclear Medicine Communications*. **32** (2), 147–154, doi: 10.1097/MNM.0b013e328341b268 (2011).
11. Goddard, E.T., Fischer, J., Schedin, P. A Portal Vein Injection Model to Study Liver Metastasis of Breast Cancer. *Journal of Visualized Experiments*. (118), doi: 10.3791/54903 (2016).
12. Limani, P. et al. Selective portal vein injection for the design of syngeneic models of liver malignancy. *American Journal of Physiology-Gastrointestinal and Liver Physiology*. **310** (9), G682–G688, doi: 10.1152/ajpgi.00209.2015 (2016).

Figure 1.

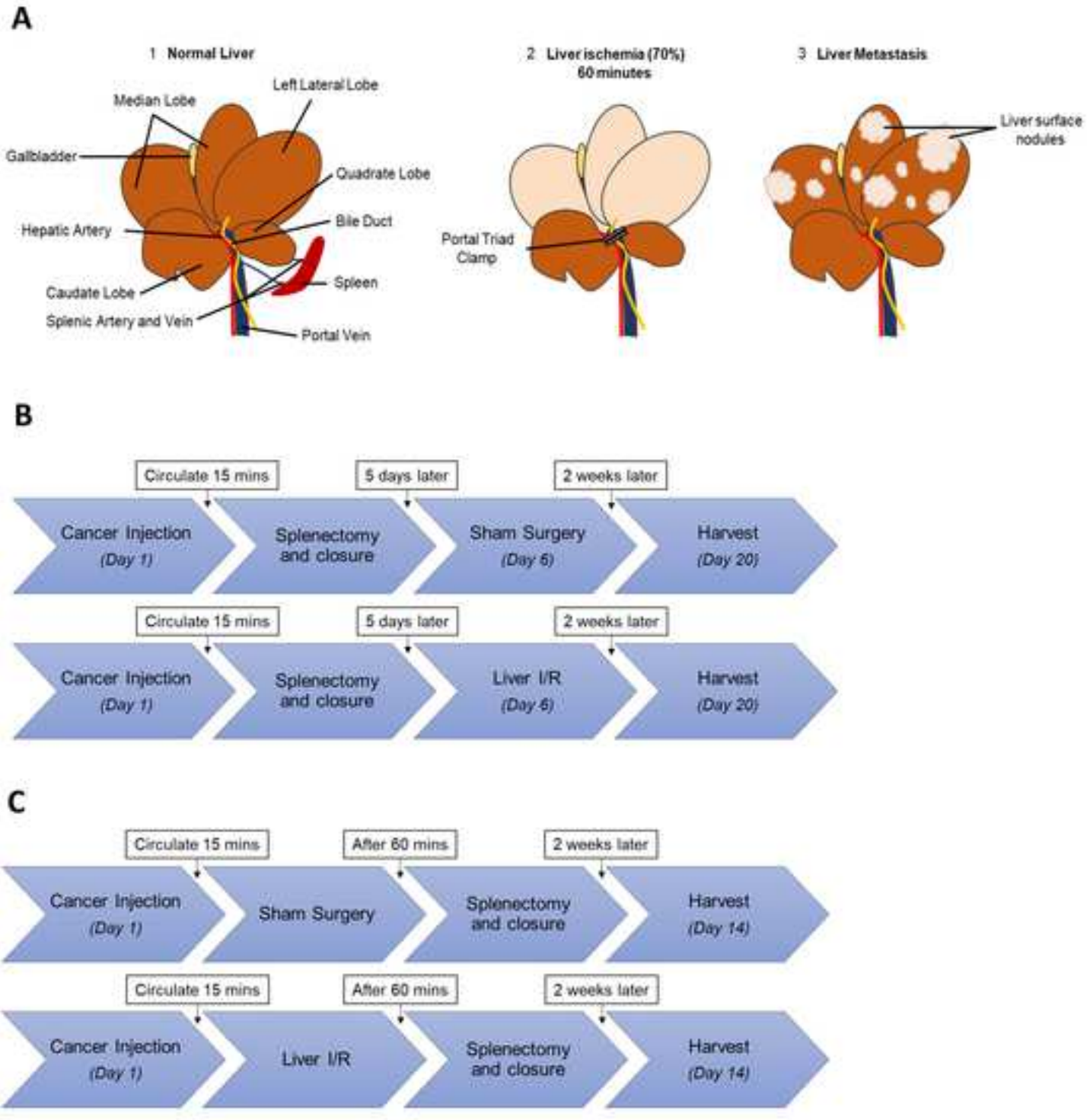
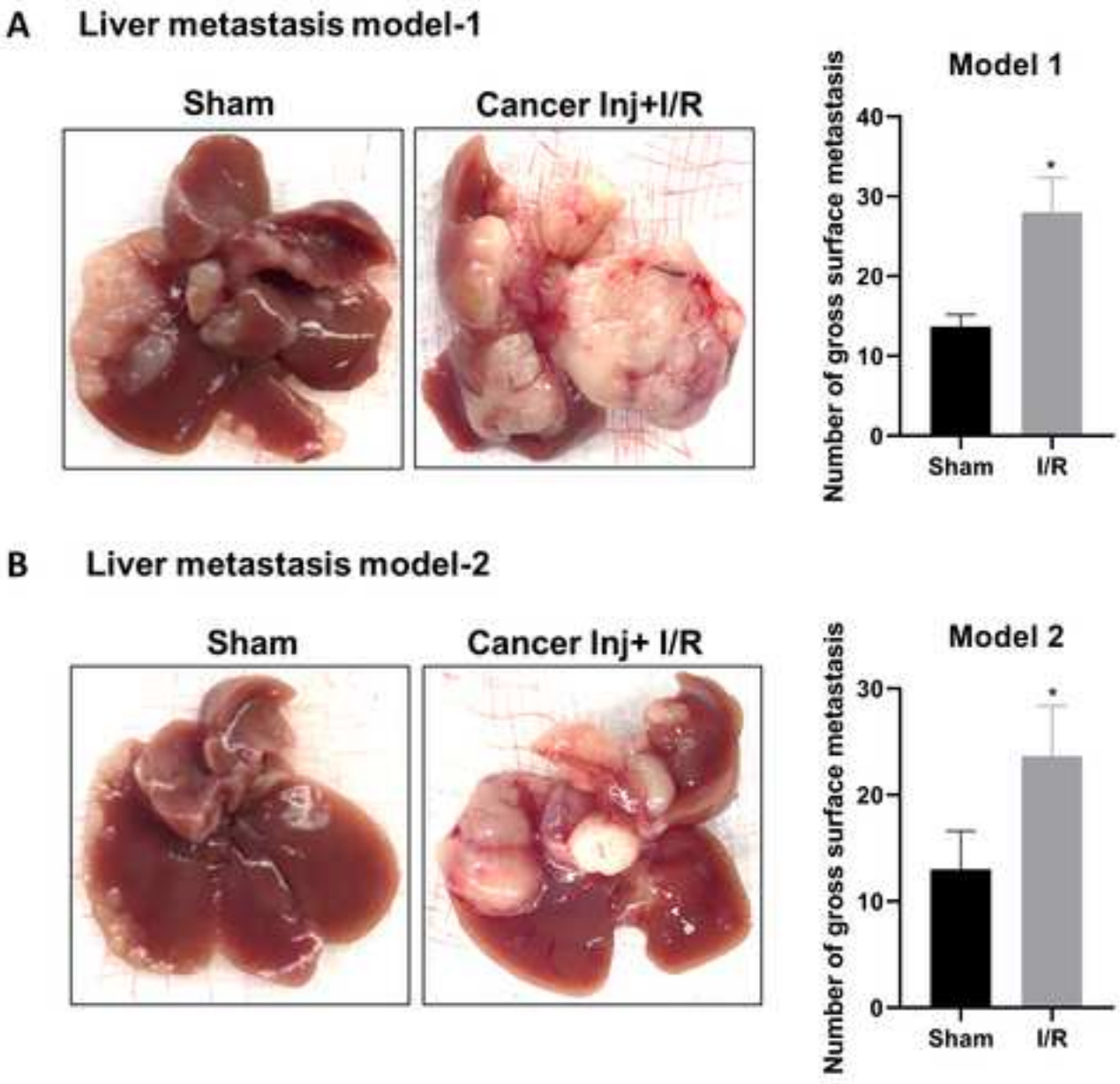


Figure 2.



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Gauze	Fisherbrand	1376152
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Micro-Serrefine clamp applicator with lock	Fine science toosl	FST-18056-14
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Micro-Adson Forceps	Fine science toosl	FST-11019-12
Micro-Adson Forceps	Fine science toosl	FST-11018-12
4-0 polypropylene suture	Ethicon	K881H
Needle holder	Harvard Apparatus	72-8826
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March 27, 2019

Dear Editorial Board,

We appreciate your interest in publishing the manuscript entitled *"A Murine Model of Metastatic Liver Tumors in the setting of Ischemia Reperfusion Injury"* to the *Journal of Visualized Experiments* (JoVE59748). We are grateful for the opportunity to resubmit a revision. The manuscript has been revised to address the supportive and constructive comments from the editor. Specific responses have been addressed point by point, changes are tracked within the manuscript and their details are described below.

The manuscript has been approved by all of the authors. The revisions are being submitted electronically as instructed. We sincerely hope that the manuscript will be accepted for publishing in its present form. The authors again wish to thank the editorial office and editor for consideration of this manuscript for publication in *Journal of Visualized Experiments*.

Sincerely,

Samer Tohme, MD
Fellow, Complex General Surgical Oncology
Department of Surgery
University of Pittsburgh

Response to Editor

Comment 1: Please check your manuscript carefully for spelling and grammatic errors. I have tried to fix several scattered errors throughout (e.g. belching used instead of blanching).

Response: We apologize for any spelling and grammatical errors. The newly revised manuscript has been thoroughly proofread and any errors have been corrected. Changes can be tracked in the attached newly revised manuscript.

Section 2. Cell culture

Comment 2-4: Add the materials to the table of materials and remove the commercial name from the manuscript.

Response: All the commercial labeling has been removed from the newly revised manuscript and the materials have been added into the table of materials file.

Section 3. Injecting tumor cells

Comment 6: Add to the table of materials. What is the ketamine dose? What is the xylazine dose? 0.1 mL/20 g for the cocktail is vague.

Response: The dosage of Ketamine and Xylazine has been described and added to the table of materials. The following sentence can be found in the newly revised manuscript.

3.1. Anesthetize 8–12-week-old male (C57BL6) mice by administering ketamine (150mg/kg) and/ xylazine (12mg/kg) intraperitoneally using a 1 mL 25 G (0.5 mm x 16 mm) needle.

Comment 7: %?

Response: The following revised sentence has been added to the newly revised manuscript.

3.5. Scrub povidone-iodine solution (7.5%) to the shaved abdominal wall to disinfect the skin before making a surgical incision.

Comment 8: Skin and muscle are both to be incised together?

Response: We apologize for the lack of detail. The commented sentence has been revised as described below.

3.6. Initially lift the skin with the tooth forceps and make a midline incision with the help of surgical scissors. Then, lift the abdominal muscle and peritoneum to create a midline incision of approximately 3 cm (midabdominal to xiphoid process) to expose the abdominal contents. Take caution not to extend the incision beyond the xiphoid process to avoid extensive bleeding.

Comment 9-10: Mention surgical tools used and explain how is this done?

Response: We have elaborated on the splenectomy procedure and added the surgical tools. The following revised sentence has been added to the newly revised manuscript.

3.14. To perform a splenectomy, use a hand-held cautery device. Carefully lift the spleen with smooth forceps and cauterize the splenic blood vessels to avoid excessive bleeding. Remove spleen by transecting the vessels at the cauterized section.

Section 4. Ischemia reperfusion Injury

Comment 11-12: How many days after 3.17? Please mention that the animal is anesthetized prior to this.

Response: A new bullet point has been added at the start of this section describing the wait period before performing the second laparotomy. The following sentence can be found in the newly revised manuscript.

4.1. 5 days after the first laparotomy, anesthetize mice by administering ketamine (150mg/kg) and xylazine (12mg/kg) intraperitoneally using a 1 mL 25 G (0.5 mm x 16 mm) needle. Follow steps 3.3-3.4.

Comment 13-14: % of Povidone-Iodine solution. Mention exact step numbers.

Response: The following revised sentence has been added to the newly revised manuscript.

4.2. Scrub povidone-iodine solution (7.5%) to the shaved abdomen of the mouse to disinfect the skin and perform a midline laparotomy as described above in step 3.6.

Comment 15: changed method to model to match fig 2

Response: We agree with the Editor's change and have included this change in the newly revised manuscript.

Section 6. Assessment of liver ischemia reperfusion injury

Comment 16-18: Define

Response: The expanded forms of the acronyms for the serum enzymes ALT, AST and LDH have been added to the newly revised manuscript in the following sentence.

6.2. Assess liver ischemia injury by measuring serum Alanine transaminase (sALT), serum Aspartate transaminase (sAST) and serum Lactate dehydrogenase (sLDH) levels. The blood can be drawn from the facial vein to extract serum 3–6 h after the initiation of reperfusion. Perform liver histology to analyze the percent tumor area within the ischemic lobe.

Comment 19-20: I added this, please check if it is okay

Response: The authors agree with the Editor's changes.

Comment 21: Mention statistical test used, sample size, and define the error bars.

Response: The following sentence has been added to the newly revised manuscript.

Results are expressed as the mean ± standard deviation. Group comparisons were performed using Student's *t* test. (n=5/group)

DISCUSSION

Comment 22 and 24: Immune competent, Blanching?

Response: We again apologize for the spelling errors. The manuscript has been thoroughly revised and the mistakes pointed out by the Editor have been corrected.

Comment 23: References?

Response: In the commented sentence here, we are referring to splenic injections and portal vein injections that have been performed in our lab. We tried both procedures to investigate which would best suit our hypothesis by yielding minimal bleeding and less chance of fatality. Nonetheless, we wanted to point out that, in our experience, both methods are valid to study liver metastases. Following articles have been cited and added into the newly revised manuscript.

9. Thalheimer, A. et al. **The intraportal injection model: A practical animal model for hepatic metastases and tumor cell dissemination in human colon cancer**. BMC Cancer. 9 (1), 29, doi: 10.1186/1471-2407-9-29 (2009).

12. Limani, P. et al. **Selective portal vein injection for the design of syngeneic models of liver malignancy**. American Journal of Physiology-Gastrointestinal and Liver Physiology. 310 (9), G682–G688, doi: 10.1152/ajpgi.00209.2015 (2016).

ACKNOWLEDGMENT

Comment 25: Any funding sources?

Response: No funding sources are required.

REFERENCES

Comment 26: Please add volume and issue numbers to 7–11

Response: The complete reference list has been updated. However, the references numbered 7 and 11 do not have allotted volume numbers.