METHODS PAGE

Retrograde Infusion of Bile Acids Into The Pancreatic Duct To Induce Pancreatitis in Rats and Mice

George Peride , Gijs JD van Acker, Johanna Laukkarinen, and Michael L. Steer

Department of Surgery#37, Tufts Medical Center, 800 Washington Street, Boston, MA 02111

e-mail: gperides @tuftsmedicalcenter.org

1. Introduction and Rationale for Models

Access to the pancreas and pancreatic tissue of patients during the early stages of clinical pancreatitis is very limited. As a result, research addressing mechanistic issues related to acute pancreatitis must be performed using experimental models of pancreatitis in laboratory animals. Although a large number and variety of experimental pancreatitis models have been described, the clinical relevance of many of those models has been questioned primarily because their induction does not rely on manipulations that replicate events thought to trigger the clinical disease.

Biliary pancreatitis is triggered by the passage of a stone or biliary sludge into or through the terminal biliopancreatic duct (1). It is the most common type of clinical acute pancreatitis but the mechanism(s) by which stone or sludge passage triggers pancreatitis has been the subject of controversy for over 100 years. In 1901, Opie suggested that the stone or sludge might trigger pancreatitis by inducing ductal hypertension as a result of pancreatic duct obstruction (8). In another report, he also suggested that the offending stone or sludge might trigger pancreatitis by allowing bile to retrogradely flow from the biliary duct into the pancreatic duct (9). Although there have been many well-done

subsequent studies by a number of investigators that support or refute each of these mechanisms, we believe that the most compelling evidence to date supports the so-called "bile reflux" theory. That support comes primarily from the observations that (a) retrograde infusion of a variety of agents into the pancreatic duct of experimental animals induces acute pancreatitis; (b) retrograde infusion of contrast material into the human pancreatic duct during ERCP can trigger acute pancreatitis; and (c) simple pancreatic duct obstruction, without retrograde flow of bile into the pancreatic duct, does not trigger severe pancreatitis in any animals tested to date with the sole exception of the American opossum (6) and, in evolutionary terms, that species is only very distantly related to humans.

In the early days of pancreatitis research, retrograde infusion of noxious agents into the pancreatic duct of various animals (dogs, cats, rabbits, pigeons, rats, etc) were the most commonly employed models of experimental pancreatitis but it was repeatedly noted that the results obtained with those models were highly variable and that the models, themselves, were difficult to control. Acute pancreatic injury in these models was noted to develop rapidly and to be very extensive. As a result, studies designed to address mechanistic issues were difficult, if not impossible.   Furthermore, the severity of pancreatitis was frequently found to be related primarily to the pressure or volume of the infusion

rather than to the nature or concentration of the infused agent and the pancreatitis that occurred was usually lethal. It appeared to be the result of either the pancreato-toxic effect or the detergent-like effect of the infused agent. Because of these concerns, the retrograde ductal infusion models of acute pancreatitis fell into general disrepute and most investigators turned to other pancreatitis models including the secretagogue (caerulein)-induced model (4), the choline deficient diet-induced model (7), and the arginine-induced model (11).

More recently, however, reliable, controllable, and more easily employed models of duct infusion-induced pancreatitis have been developed for use with either mice or rats. Initially, those models were used for experiments employing rats but, recently, our group (5, 10) has described a modification of that rat model which allows for its use in mice and, in this way, permits experiments employing the wide array of readily available genetically modified mice. Because we have more extensive experience using the mouse model, we will begin this contribution to Pancreapedia by providing a detailed description of that model and we will then provide a more limited and focused description of the rat bile acid-induced model. The rat model which we have employed was described by Nevalainen and co-workers (2, 3) while the mouse model which we developed for mice was adapted from that rat model.

Based on our present understanding of clinical biliary pancreatitis, we currently believe that these bile acid-induced models of pancreatitis are ideally suited for use in studies designed to probe mechanistic issues related to pancreatic injury in biliary pancreatitis. They (a) are induced by a mechanism which **may** be responsible for the triggering of the clinical disease (i.e. reflux of bile into the pancreatic duct), (b) lead to development of highly reproducible, titratable, but non-lethal injury, (c) cause injury that resembles the injury noted in clinical biliary pancreatitis (i.e. lobular as opposed to diffuse and homogeneous injury) and (d) can be adapted for in-vitro studies (i.e. exposure of isolated acini to bile acids). On the other hand, these bile acid-induced models are not ideally suited for studies designed to probe issues related to non-pancreatic events (i.e. lung injury, sepsis, etc) in pancreatitis because they mandate anesthesia and open surgery which,

itself, can lead to those events thus making interpretation of results complicated as well as difficult.

2. Materials

A. Animals and reagents
1. Mice (25-30 gm, 8-12 weeks old) of either sex and any strain. (Note 1)
2. Ketamine (100 mg/ml); Xylazine (100 mg/ml)
3. Buprenorphine (0.3 mg/ml)
4. Betadine (povidine iodine) 5%
5. Sterile saline (0.9%)
6. Evans Blue (100 mg/ml)
7. Bile acid (Note 2)

B. Equipment
1. Needles (25 and 30 G 1/2 inch)
2. Sutures/ligatures (5-0 and 7-0 prolene blue monofilament taper cut needles, 8-0 and 10-0 prolene with spatula curved needle)
3. Assorted small surgical instruments
4. Infusion pump (Harvard Apparatus Model 702211)
5. Tubing (PE10)
6. Dissecting microscope and lamp
7. Warming pad (Note 3)

C. Reagent Setup
1. Ketamine/Xylazine solution: add 1 ml xylazine (100 mg/ml) to 10 ml ketamine (100 mg/ml). Can store at 4 ^{0}C for 1 month. Dilute 1-to-10 in saline before use.
2. Bile acid/saline solution: Prepare a fresh stock solution of bile acid (selected type and concentration, see Note 2) in sterile saline on day of surgery
3. Bile acid Evans blue solution: add 1 part Evans blue solution to 99 parts bile acid solution on day of surgery

D. Equipment Setup
1. Cannula: Prepare the cannula by connecting a 30 G needle to a 20- to 30-cm long segment of PE10 tubing. This end of the cannula will initially be connected to a fluid-filled syringe (without trapped air bubbles) but, eventually, it will be connected to the infusion pump.

Then, remove the plastic hub from another 30 G needle. Cut the metal portion to a length of about 1-1.5 cm and blunt both ends of this metal portion by filing. Insert one end of this filed metal needle into the PE10 tubing. The other end of this blunted needle will, eventually, be inserted into the biliopancreatic duct.

2. Infusion pump: fill the syringe so that no air bubbles are trapped in it. Mount the syringe to the infusion pump and run it a 0.05-0.5 µl/min to avoid saline precipitation at the tip of the needle (Note 4)

3. Methods

I) Retrograde Infusion of Bile Acids into the Rat Pancreatic Duct to Induce Acute Pancreatitis (9).

A. **Anesthesia:** Administer 14 µl ketamine/xylazine solution per gram body weight by intraperitoneal injection (i.e. 125 mg/kg ketamine and 12.5 mg/kg xylazine. Monitor adequacy of anesthesia by pinching the toe using forceps. Adequate anesthesia is characterized by the lack of a withdrawal response to pinch.

B. **Shave chest and abdomen** with electric trimmer

C. **Positioning of mouse:** place the animal on its back on the surface of a plexiglass sheet that has been cleaned with 70% ethanol or Cylodex and placed on a 37 ^{0}C warming pad. Immobilize the animal on the plexiglass using surgical tape

D. **Cleanse and disinfect** the shaved area by wiping it 3 times with betadine-soaked gauze pads and 70% alcohol-soaked pads.

E. **Laparotomy**: make a midline laparotomy (1.5 cm) extending from the xyphoid towards the umbilicus

F. **Exposure:** pull the liver upward (toward the head of the mouse) and away from the intestine with a small self-retaining retractor. Hold the retractor in that position by resting it on a 1 cm cushion on the plexiglass so that it is at the same height as the body of the mouse.

G. **Identification and rotation of the duodenum:** The duodenum can be seen just below the right side of the liver (on the left as the mouse is viewed). It is the first part of the small intestine, at the distal end of the stomach. "Flip" the duodenum over so that the back (i.e. dorsal) side of the duodenum is seen. In this position, the biliopancreatic duct can be seen on the liver side of the duodenum, and its junction with the duodenum will appear white. (Note 6).

H. **Immobilizing the duodenum:** place traction sutures to hold the duodenum in this position. To accomplish this, place a 7-0 or 8-0 prolene suture in the duodenum just to the mouse's left side of the papilla. It should be placed perpendicular to the axis of the duodenum and it should grasp at least half of the circumference of the duodenum, avoiding any blood vessels. If necessary, place a second traction suture near the mouse's right side of the papilla. Grasp and hold the ends of the traction sutures using small needle holders which are then allowed to rest on the plexiglass platform so that their weight exerts continuous traction on the sutures and holds the duodenum in its desired position. Once the duodenum is fixed in this desired position, one should have a clear view of the back side of the duodenum, the duodenal portion of the pancreas, the distal biliopancreatic duct, and the region of the papilla. This view should be maintained without the need for any further manual distraction or handling of the tissues so while also allowing the operator to work with both hands.

I. **Cannulating the duct:**

1. Place a 10-0 proline suture around the biliopancreatic duct as close as possible (i.e., within 1 mm) to the edge of the duodenum. It may help to stretch and lift the duct slightly so that it is exposed better. It is important not to encircle any pancreatic tissue in this suture, because that portion of the gland would probably be injured as a result. It may be helpful to drip water or saline on the tissues at this point as it will enable the pancreatic tissue to slide away from the duct.

2. After the suture has been placed, make a large loop out of the two ends of the ligature using a microsuturing needle holder so that the ligature can be easily tied down once the cannula is in place. Until that time, the ends of the suture can

be left on top of the duodenum so that they can be easily located later.

3. Lift the duodenum slightly in the direction of the liver and then place a curved or straight microclamp (bulldog clamp) on the bile duct close to the hilum of the liver (Note 7). This will prevent the infusate from flowing into the liver and/or gallbladder.

4. Puncture the wall of the duodenum directly opposite to the papilla using a 25 G needle. The needle should follow the same angle as that followed by the duct as it enters the duodenum.

5. Turn the pump off and clean the cannula to be sure that there is no air in the cannula which could lead to an "air lock" and obstruction to the biliopancreatic duct later.

6. Hold the cannula using serrated microdissecting tweezers and pass it through the puncture hole in the duodenum with its blunted, beveled tip pointing upward. It should pass easily through the puncture wound. Then "scrape" the wall of the duodenum near the papilla using the cannula until the papilla is found. Once the papilla is reached, **gently** press the cannula upward toward the liver so that it passes through the sphincter and enters the duct for ~2 to 3 mm. Then tilt the cannula so that it lies parallel to the duct and it can be seen in the duct above the edge of the duodenum.

7. Tighten the previously placed knot in the 10-0 prolene suture around the duct to stabilize the cannula.

8. Slowly withdraw the cannula so that it is no more than 2 mm past the sphincter on the liver side of the knot.

J. **Infusion:** Turn on the pump and infuse the selected fluid (saline or saline containing bile acid and blue dye) at a flow rate of 5 µl/min for 10 min. We avoid flow rate higher than 10µl/min to avoid artifacts and injury due to rapid increase in intrapancreatic pressure. Throughout the infusion, keep the gut moist using saline and monitor the appearance of the duct and pancreas. If the cannulation and infusion have been successful, there will be scattered blue spots in the pancreas (mostly in the head of the gland), but no localized or diffuse swelling of the pancreas or blue staining of the tissues around the duct. These latter signs would indicate that the duct has been disrupted or that the cannula is not in the duct and the procedure should be aborted. If fluid accumulates around the duct, blot it using a Q-tip to see whether it is blue; if so, the the procedure should be aborted. If high concentrations of bile acid are being used intentionally, blue blebbing of the pancreas might be seen even if the cannula has been properly placed, in which case the procedure might not need to be aborted.

K. **Removal of the cannula and microclamp after completion of the infusion:** Gently remove the cannula from the duct by gently pulling the cannula using micro-dissecting serrated tweezers. Then, use micro-dissecting spring scissors to cut the securing 10-0 prolene knot. Cutting the knot should be done very carefully so as not to injure or perforate the underlying duct. Using a Q-tip, test to see whether there is blue dye coming out of the pancreas. If this is the case, then the animal should be discarded. After the cannula has been successfully removed, the microclamp occluding the biliary duct, described in the section "cannulating the duct", should be removed by squeezing and tilting it backward with forceps.

L. **Closure:** Suture the hole in the duodenum with a 10-0 prolene suture. Place this suture parallel to the intestinal axis and ~2 diameters (of the hole) to the left and right side of the hole. Release the traction sutures and flip the duodenm back to its normal position. Suture the peritoneum with a 6-0 prolene suture using a running stitch. Suture the skin in the same way with 5-0 or 6-0 prolene suture or using 7 mm Michelle wound clips.

M. **Postoperative analgesia:** Give a single dose of buprenorphine (0.05-0.1 mg/kg) during the infusion or immediately after wound closure.

N. **Recovery:** return the mouse to its cage placed on a heating pad (37 ^{0}C) until it recovers. Then give the mouse food and water ad libitum.

Anticipated Results

With practice and gentle surgical technique, >95% of the mice can be successfully infused and, over the week following infusion, >95% of successfully infused mice will recover completely. When evaluated 1 week after infusion, the pancreas will show little or no evidence of earlier pancreatitis. Pancreatitis, elicited by infusion of either Na-taurocholate (2%, 37 mM, 50 µl) or TLCS (0.18%, 3 mM, 50 µl) will be maximally severe 24 hours after bile acid infusion but infusion of only saline will cause little or no evidence of pancreatic injury at this time.

We do not discriminate between acinar cell injury and acinar cell death because no clear distinction can be made between the two on purely morphological grounds. It is important to include control (i.e. saline-infused) mice in each experiment to allow for accurate interpretation of the morphological appearance of the pancreas following bile acid infusion since, even in non-operated mice, some of the acinar cells may appear to be either injured or dead. We have presumed that this low background "noise" level of apparent injury results from some unavoidable staining artifact.

Our standard protocol involves infusion of 50 µl of 3 mM TLCS in saline solution. Mice are sacrificed 24 hours after bile acid infusion and the results obtained following infusion of the bile acid are compared with those obtained following infusion of saline alone. The parameters that we use to quantify pancreatitis severity include measurement of plasma amylase activity, pancreatic edema (i.e. water content expressed as percentage of wet weight), acinar cell injury/necrosis (quantified by morphometric analysis of H&E-stained sections) and pancreatic inflammation (i.e. pancreas myeloperoxidase contents). Each of these parameters of pancreatitis severity are markedly and maximally elevated 24 hours after TLCS infusion but little or no evidence of pancreatitis is noted after infusion of saline alone. Pancreatitis is most severe, and mostly limited to, the head portion of the gland

and only minimal changes are noted in the tail. Minimal or no evidence of pancreatitis is observed in the head of the pancreas after infusion of only saline (Laukkarinen et al, Gut 2007).

The severity of pancreatitis is highly dependent upon the concentration of the bile acid infused and the volume of the infusate (5). Typically, the distribution of pancreatic injury in this model of pancreatitis is lobular rather than diffuse and normal-appearing lobules will be noted adjacent to clearly injured lobules.

The mouse common biliopancreatic duct is quite short and, after clipping the bile duct at the liver hilum and positioning and fixation of the infusion cannula, most if not all of the infusion solution passes retrogradely into ducts supplying only the head portion of the gland. As a result, the changes of pancreatitis which follow bile acid infusion are limited to the pancreatic head. Accordingly, we limit our observations to changes that occur in the pancreas within ~5 mm of the duodenal sweep and we do not monitor changes that may occur within other areas of the gland.

II) Retrograde Infusion of Bile Acids into the Rat Pancreatic Duct to Induce Acute Pancreatitis

The basic maneuvers for eliciting bile acid-induced pancreatitis in rats are identical to those employed when bile acid-induced pancreatitis is elicited in mice. In this section, we will limit our description to those maneuvers which are specific to the rat model.

MATERIALS specific to rats
1. Rats (150-200 g, 10-15 weeks old) of either sex and any strain are used. In theory, larger or smaller rats can also be used.
2. Anesthetic: Prepare a stock solution of ketamine/xylazine by adding 1 ml of xylazine (100 mg/ml) to 10 ml ketamine (100 mg/ml). On the day of surgery, inject i.p. 10 µl/g rat of the stock solution for a final dose of 100 µg/kg ketamine and 10 µg/kg xylazine.
3. Infusion marker: Prepare a stock solution by dissolving Evans Blue in saline at a final concentration of 100 mg/ml and, at the time of surgery, add 1 part of Evans Blue solution to 99 parts of bile acid solution.

Procedures

A. Exposure: After delivering the duodenum from the depths of the wound, the biliopancreatic duct can be seen on the surface of the pancreas. However, as it approaches the duodenum it dives down and is covered by pancreatic tissue. One has to look carefully at the duodenum about 2 cm from the end of the stomach to see the biliopancreatic duct entering the duodenum. Unlike the mouse in which the papilla is clearly visable and white, lobules of the pancreas cover the duct in the rat and it can not be clearly seen at this point.   If the duodenum is fixed in this position with traction sutures (6-0 or 7-0 prolene), the surgeon can work comfortably and without repeatedly grasping or handling the head of the pancreas.   This is important because it permits the operator to avoid traumatizing the pancreas and duodenum during the process of duct cannulation.

B. Cannulation of the duct: Place an 8-0 or 10-0 prolene suture around the bilio-pancreatic duct as close as possible (i.e. within 2 mm) to the edge of the duodenum. It helps to stretch and lift the duct slightly so that it is exposed better. As the papilla is not as clearly seen as in the mouse, the biliopancreatic duct appears yellow-gray in color. It is inevitably covered by a small portion of the pancreas and, because of this, a small portion of the pancreas will usually be tied and injured in the process of fixing the cannula in position. The 8-0 or 10-0 prolene suture ends are difficult to see and they can easily be lost. To prevent this from happening, keep them constantly attached to microsuturing needle holders until the ends are tied after the cannula has been placed.

C. Infusion: Turn on the pump and infuse the selected fluid (saline or saline containing bile acid and Evans Blue) at a rate of 50 µl/min for 10 min.

D. Closure: Suture the hole in the duodenum closed with a 10-0 prolene suture. Suture the peritoneum closed with a 6-0 or 7-0 prolene running suture.   Suture the skin closed the same way with a 5-0 or 6-0 prolene suture or close it using 7 mm Michelle wound clips.

E. Monitoring the severity of pancreatitis: Because the rat common biliopancreatic duct is longer than in the mouse, more of the pancreatic ducts will be retrogradely infused and pancreatitis will be noted in a larger portion of the gland.   As in the mouse, however, pancreatic injury 24 hours after infusion will have a lobular distribution.

4. Notes

Note 1: Although we usually use mice that weigh 25-30 g, larger or smaller mice can also be used. The severity of pancreatitis induced by this method depends, to a variable extent, on the mouse strain being used.   For this reason, experimental and control mice should be of the same strain and, if possible, experiments using genetically modified mice should use wild-type littermate mice as controls. As the size of the mouse affects the size of the pancreas, mice of the same size should also be used. When saline-infused mice are used as controls, they are treated in exactly the same way as the experimental mice, but in case of control animals, the infusate consists of saline only. Infusion of saline alone causes a mild injury that is transient and resolves over the subsequent 24 hours. During training, the surgeon should also include control mice subjected to sham operation, which includes handling of the relevant tissues but no cannulation, in order to be certain that this does not result in any pancreatic injury/inflammation.

Note 2: In principal, most bile acids could be used, but it would be best to use a naturally occurring one.   To reduce the likelihood of detergent- or ionophore-like bile acid effects, a sub-micellar concentration of the bile acid should be infused and the concentration of the bile acid should be as low as possible. Our experience is limited to the use of either Na-taurocholate or Na-taurolithocholate 3-sulfate (TLCS). We have obtained almost identical results with both of these bile acids although the concentrations needed to elicit pancreatitis with these 2 bile acids differ considerably. If other acids are chosen by the experimentalist, preliminary studies should be performed to confirm the fact that their infusion elicits pancreatitis and to define the time and concentration dependence of that response.

Note 3: It is important to place the mouse on a warming pad to prevent hypothermia, especially the worsened hypothermia which can occur when

an anesthetized animal is subjected to laparotomy and exteriorization of bowel.

Note 4: It is important to ensure that no air bubbles are trapped in the infusion apparatus because trapped air bubbles can create an air lock, interfering with infusion. Air bubbles trapped in the duct after removal of the cannula can also interfere with ductal drainage. The easiest was to be sure that bubbles of air are not trapped in the infusion system is to first fill the syringe, then attach the tubing, and then press the tubing to remove air.

Note 5: It is important to stress the need for gentle handling of all tissues during this procedure. Handling the tissues in a rough manner will induce injury which may be confused with pancreatitis and/or promote morbidity or mortality of the animal. With gentle operative technique, >95% of infused animals will survive and the pancreas will appear grossly normal 1 week after infusion.

Note 6: The duodenum must be handled gently using serrated tweezers when it is being "flipped" in order to prevent injury to it and to the pancreas. When grasping the duodenum to "flip" it, one should grasp at least half of its circumference as this will cause less injury than grasping and pinching just a small part of it. Care must also be taken to avoid stretching or pulling the duodenum vigorously as this would also stretch and distort the biliopancreatic duct.

N.B. At this time and at all later times during the operative procedure, the remainder of the intestine must be left inside the abdominal cavity so that it remains warm and moist. Allowing the intestine to come out of the abdominal cavity will cause significant heat loss to the animal as well as drying of the tissues. If drying of tissues is noted, gently moisten the tissue with warm saline.

Note 7: Avoid clamping the portal vein with this clip. If this happens, the duodenum will swell and turn purple and the procedure may need to be aborted unless the microclamp can be properly repositioned.

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